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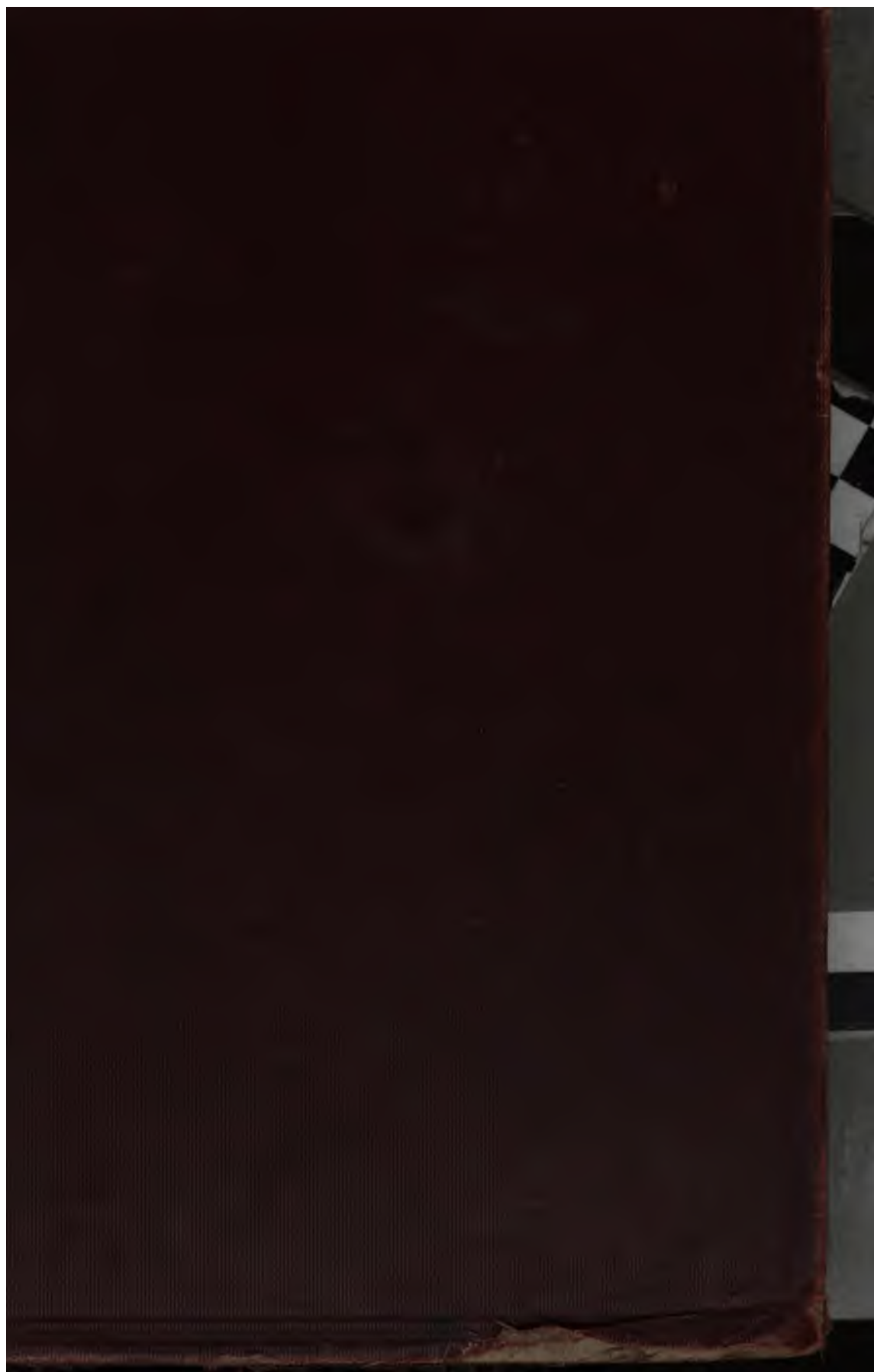
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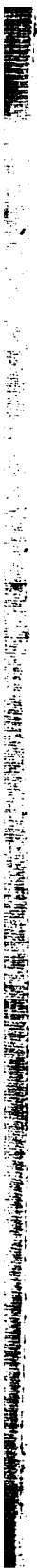
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**THE PRINCIPLES OF
IMMUNOLOGY**

THE PRINCIPLES OF IMMUNOLOGY

UNIV. OF
CALIFORNIA

BY

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TO
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WILLIAM C. KARSNER
CHARLES W. KARSNER
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DOCTORS OF MEDICINE

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PREFACE

THIS book has been prepared in the hope that a concise statement of the facts and more important hypotheses concerning resistance to infection may serve to provide a clear understanding of a subject of the utmost importance in modern diagnosis and treatment. Designed primarily for students of medicine and for those practitioners whose duties have made it impossible to digest a large mass of publications on the subject, the scope of the book is restricted to fundamental principles. The plan throughout is to present on an experimental basis the demonstrated facts and to supplement these with brief discussions of the practical and theoretical bearing of the phenomena upon resistance and disease in man. A few illustrations have been inserted, but it must be recognized that technical details can only be fully comprehended on the basis of actual work with the methods. The usual diagrams of the side-chain theory have been omitted because of the belief that they serve to confuse rather than clarify the conception of processes whose fundamental basis lies in the field of physical chemistry. Certain material concerning the practical application of immunology to the prevention and cure of disease has been collected in three appendices. These have been added in order to explain the basis of the practical methods rather than as an exact guide in their application.

Knowledge progresses from the known to the unknown, from the simple to the complex, and if the brevity of the book serves to implant essentials in such a way that the reader not only grasps the facts, but finds himself stimulated to seek further information and discussion in more comprehensive works, the most compelling aim of this book will have been achieved. For this purpose books which we have used with considerable freedom are recommended: Zinsser, "Infection and Resistance"; Wells, "Chemical Pathology"; Kolmer, "Infection, Immunity, and Specific Therapy"; Kraus and Levaditi, "Handbuch der Technik und Methodik der Immunitätsforschung"; Muir, "Studies on Immunity"; Kolle and Wassermann, "Handbuch der pathogenen Mikroorganismen"; Metchnikoff, "Immunity in Infective Diseases"; Bordet, "Traité de l'Immunité dans les Maladies Infectieuses"; Besredka, "Anaphylaxis and Antianaphylaxis"; Bordet and Gay, "Studies in Immunity"; Gay, "Typhoid Fever"; Browning, "Applied Bacteriology"; Craig, "The Wassermann Test"; Noguchi, "Serum Diagnosis of Syphilis"; Zinsser, Hopkins, and Ottenberg, "Laboratory Course in Serum Study." The names of those who have contributed to the literature are given in the text, but precise references have been omitted, since the articles can be found by reference to such bibliographic journals as the *Index Medicus*, *The Index*

Catalogue of the Surgeon General's Office, and in particularly available form in the *Quarterly Cumulative Index of the American Medical Association*. Every effort has been made to give credit where it belongs; if omissions or errors have been made they are due to the vast amount of material that has been accumulated on this subject rather than to intentional oversight.

Our thanks are due to our colleague, Doctor Maurice L. Richardson, for extremely valuable aid in the revision of the manuscript, to Mr. E. L. Miller for three important microscopic drawings, to Miss May E. Treter and Miss Catherine E. Lennon for faithful and painstaking clerical work. Mr. W. T. Brownlow, of Cleveland, has made the line drawings and Mr. E. F. Faber, of Philadelphia, the drawings of the lungs in anaphylactic shock. We have taken materials from certain journals and make grateful acknowledgment by reference in the text.

January, 1921.

HOWARD T. KARSNER,
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INTRODUCTION

THE history of immunology as a science is distinctly modern and in the investigation of details dates back only as far as the time of Louis Pasteur. Jenner's work on smallpox vaccination represents most painstaking and thorough investigation; it was epochal in character, and of the utmost importance in practical results, but was not immediately followed by any general application to other diseases, probably because of the limitations of technical methods. Observations of the phenomena of immunity were, however, made in ancient times and the resistance to second attacks of such diseases as measles, scarlatina, variola, varicella must have been common knowledge from the earliest days of the human race. Whilst many of the earlier students of medicine recognized a certain similarity between poisoning and infectious disease, yet Hippocrates could see no such resemblance and his theory of the four humors was dominant throughout the Middle Ages. With minor exceptions this belief held sway until well into the Renaissance. In 1548, however, Fracastore proposed the theory that infection was carried from person to person "per contactum" or "per fomites," and from this time dates real progress in the investigation of infectious disease. This led subsequently to the establishment of two schools of thought, the one believing disease to be due to substances of basic or acid principle, and the other believing disease to be due to parasites. The development of the latter idea was forced to await the discovery of means to view minute parasites and, as a matter of fact, was delayed much longer, because the invention of the microscope by Kircher in 1659 and van Leeuwenhoek in 1675 far antedated the connection now established between minute parasites and infectious disease. Nevertheless, Plenciz in 1762 expressed a belief in the direct etiological connection between certain forms of disease and microorganisms, and established the conception of the "contagium vivum." This idea was revived by Henle and by Brettoneau, but attracted no permanent attention.

As perhaps the first observation leading up to our present conception of infectious diseases, and therefore to immunity against them, was the discovery in 1837 by Schwann that certain forms of fermentation are due to the presence of yeasts, an observation made at about the same time by Cagniard-Latour. Although at this time there was little, if any, thought that this discovery had any important bearing on infectious disease, yet within the succeeding decade favus, thrush, and pityriasis versicolor had been demonstrated to be due to specific fungi. Nevertheless, the possible similarity of fermentation and infectious disease had been considered in a more or less philosophical way, and Robert Boyle had said: "He that thor-

oughly understands the nature of ferments and fermentations shall be much better able than he that ignores them to give a fair account of diverse phenomena of certain diseases (as well fevers as others), which will perhaps be never properly understood without an insight into the doctrine of fermentations." In the further development of the origin of infectious disease in living organisms perhaps the work of Rayer and Davaine on anthrax was of the utmost importance. They reported in 1850 that in the blood of anthrax victims "are found little thread-like bodies about twice the length of a blood-corpuscle. These little bodies exhibit no spontaneous motion." In 1863 Davaine showed that the blood containing these rods could transmit the disease while blood free from them did not transmit the infectious agent. Davaine suggested at this time that the manifestations of the disease might represent the results of the specific fermentation produced by these bacilli. Such a parasitic conception of disease was further supported by the discovery in 1873 of the spirillum of relapsing fever by Obermeier. Subsequently the work of Louis Pasteur, Koch, and the great school of early bacteriologists gave the final evidence in support of the "contagium vivum."

Although the essential development of the science of immunology necessarily awaited the critical study of infectious disease, as can be seen from the foregoing summary of the development of the knowledge of the cause of infections, yet throughout the ages there had been speculations as to the nature of immunity running hand in hand with hypotheses as to the nature of infection. Immunology took its most important step forward more than a half century before the work of Schwann had reached its fruition in the studies of Davaine, Obermeier, and Pasteur; namely, in the masterful experiments of Jenner. It is almost certain that for at least a century before Jenner's publication there had been practised, in the far and near East as well as in certain parts of Europe, including England, the inoculation of smallpox during full health in order to produce a mild attack of the disease and thus protect against later more severe or fatal attacks. It is indeed possible, as claimed by Carburi, that such a procedure originated in Europe as early as the sixteenth century and was carried to Constantinople and thence to the far East. Similar attempts to produce mild attacks of other diseases were tried, but with little success, as, for example, the work of Vesepremi in 1755 with plague, of Home in 1757 with measles, and of Turenne in 1844 with syphilis. It seems unlikely, however, that any of this work had any direct bearing on the discovery of Jenner. Sprengell states that for many years before Jenner's time the protective influence of cowpox against smallpox was known in certain districts of Ireland, Holstein, Brandenburg, Switzerland, Catalonia, Peru, and the East Indies. Similar observations had been published, as, for example, the statement of Böse in 1769, that persons who had suffered cowpox were not subsequently attacked by smallpox. Jesty in 1774 had inoculated some members of his family with cow-

pox and reported that they remained free from smallpox. In 1791 Jensen and Plett practised protective inoculation with cowpox and reported good results, as did also Penster in 1765. None of these studies, however, bore critical scientific examination, nor did they serve to stimulate active work along this line. Indeed, it seems unlikely that they influenced Jenner in any way. Jenner brought to bear the critical method of the experimental investigator and proved the point. The method was rapidly put into clinical practice, spread over the British Isles and Europe and stood the test of time and wide application. With very slight modifications it stands to-day, in spite of our great advances in the study of immunity, as the most effective method we have to guard against infectious disease. Jenner vaccinated a boy on the arm with cowpox virus obtained from a lesion on the hand of a dairy maid, and subsequently inoculated the boy with fresh smallpox virus, which failed to produce the disease. He also reported an attempt to inoculate smallpox unsuccessfully in ten persons who had had cowpox nine months to fifty-three years previously. In 1800 Waterhouse in Boston repeated the experiment of Jenner on his own son, and in 1802 performed a more extensive and even more critical experiment, in which he vaccinated nineteen boys with cowpox. Twelve were inoculated with smallpox virus three months later and failed to develop the disease, the same virus being inoculated at the same time into two unvaccinated boys, producing well-developed smallpox. ~~The virus from these latter two boys was later inoculated into all the nineteen vaccinated boys without results.~~ Thus began the period of experimental investigation of the phenomena of immunity. Further progress of importance was not made until 1880, when Pasteur announced his results in vaccination against chicken cholera. No brief review such as this can do justice to the stimulus to modern biological science furnished by this man and his associates, and the reader is referred to the interesting and intimate view of the life of Pasteur written by Valery Radot, his son-in-law. At the beginning of Pasteur's work the theory of spontaneous generation was still generally accepted by the scientific world, and before he was compelled to cease his active investigations not only had this theory been overthrown, but also the ideas of chemists in regard to crystallization and to the rotation of light by bodies in solution had been completely revised, the silk and wine industries of France, and indeed of the world, had been entirely rejuvenated, the bacteriological cause of numerous diseases conclusively proven, and the science of immunology put on a plane where its progress must be uninterrupted. His first contribution to the science of immunology was in connection with his work on chicken cholera. Although he did not offer it as such, nevertheless, this incident well illustrated his doctrine that "chance favors the prepared mind." He had saved some old cultures of this bacterium and later found that they were avirulent. He subsequently tried to cause the disease in

animals which had been inoculated with this virus, using the second time a culture which was virulent for untreated fowl. He showed that the inoculated fowl were immune to the virulent culture. In 1881 he demonstrated with his collaborators, Chamberland and Roux, that this was not an isolated fact, but that essentially the same thing had been accomplished with anthrax. The virus of anthrax could not be attenuated by the same simple method as for fowl cholera, because the bacillus anthracis preserves its virulence by the formation of spores. They showed, however, that they could prevent the formation of spores by growing the bacillus at 42° to 43° C. At this temperature growth of six to eight days sufficiently attenuates the organism for protective inoculation. The proof of the vaccination was given publicly before the Society of Agriculture at Melun. For this phenomenon Pasteur used the term vaccination, and in London in 1881 said: "I have lent to the expression vaccination an extension that I hope science will consecrate as a homage to the merit and immense services rendered to humanity by one of the greatest men of England—Jenner." In 1882 Pasteur and Loir confirmed Thuillier's observations on the cause of swine fever and then successfully vaccinated pigs against this disease. Then in 1885–1886 came the final brilliant chapter in the work with rabies, in which vaccination was practised without definite knowledge of the etiological agent. The work with rabies was of further importance in that it led to the discovery of the fact that a virus may be increased in virulence, a phenomenon quite the reverse of the earlier discovery of the possibility of attenuation.

In his studies Pasteur had worked almost entirely with the active organisms causing disease, and the next step forward was the discovery that the products of bacterial growth and activity can be utilized in the development of immunity. Salmon and Theobald Smith published in 1886 their studies on the immunization of hogs against hog cholera by the use of the products of the specific organisms. This idea had been suggested by Loeffler in 1884, but not proven. Before he had made any conclusive experiments the subject had been taken up by numerous other investigators. Behring and Kitasato in 1890 had discovered tetanus toxin and Roux and Yersin in 1888–1889–1890 had published their discovery of diphtheria toxin. These workers showed that the symptoms of the special diseases studied could be reproduced by the soluble products of the causative organisms and by their later work that one of the important phases of immunity is due to the development of substances capable of neutralizing these products. It became evident with further work that this principle does not apply to all pathogenic organisms, and the work of Pfeiffer with cholera in 1891 led to the differentiation of exotoxins and endotoxins.

The antagonistic action of blood and body fluids on putrefaction had been noted by John Hunter, Traube, and Lister, but Grohman in 1884 was the first to publish well-founded experiments upon the

inhibition by fresh plasma of the actual growth of bacteria. Flügge and Nuttall in 1888 demonstrated under the microscope the destruction of bacteria by blood, and Buchner in 1889 showed this property to be present in the serum. At about the same time the work of Richet and Héricourt and of Babes and Lepp showed that an immunity artificially produced against pyogenic cocci and against the virus of rabies could be transferred from one animal to another by means of the blood serum. These studies were followed almost immediately by the discoveries of Behring and Kitasato that the serum of animals immunized to the toxins of tetanus and of diphtheria bacilli not only could produce immunity in other animals, but that the specific disease could be cured by the use of the respective sera. These discoveries led immediately to the development of serum therapy, and in 1894 diphtheria antitoxin was being marketed in Germany. Contemporaneously with these developments Metchnikoff conducted his observations and experiments upon phagocytosis, and in 1883 published his "*Récherches sur la digestion intracellulaire.*" He studied various lower forms of life, such as echinoderms, and found that during metamorphosis the atrophic cells of the larvæ are devoured by other cells, either leucocytes or other phagocytic cells. These studies were later extended to include reparative conditions, such as the healing of wounds and resistance to infection. The outcome was a series of brilliant discoveries of the part phagocytosis plays in combating bacterial invasion, and ultimately the practical application in the use of bacterial vaccines for prevention and treatment of infectious disease. The discovery of the various forms of immune bodies and of the substances which might lead to the production of such immune bodies followed with considerable rapidity, but the details may best be left to the study of the particular immune bodies concerned, which include agglutinins and precipitins, cytoly-sins, and other complement binding substances. "That a plague of diarrhea in a poultry yard, studied by a professor of chemistry, should be the seed from which has grown the vast development of later years is a strange fact, but fact, nevertheless" (Adami).

THE PRINCIPLES OF IMMUNOLOGY

CHAPTER I

VIRULENCE OF ORGANISMS

MUTUAL RELATIONS OF PARASITE AND HOST.

PARASITISM.

VIRULENCE.

METHOD OF DEMONSTRATION.

THE BASIS OF VIRULENCE.

ANIMAL PASSAGE.

CAPSULE FORMATION.

AGGRESSINS.

POISONOUS SUBSTANCES OF BACTERIAL ORIGIN.

PTOMAINS.

TRUE TOXINS (EXOTOXINS).

ENDOTOXINS.

POISONOUS BACTERIAL PROTEINS.

ALTERATIONS OF VIRULENCE.

INCREASE OF VIRULENCE.

DECREASE OF VIRULENCE.

Mutual Relations of Host and Parasite.—The existence of infectious disease depends fundamentally upon the invasion of a plant or animal by some infective agent. The infective agent is usually a microorganism either bacterial or protozoan in nature, although infestations by larger organisms, such as worms within the body or various forms of pediculi upon the surface, are sometimes spoken of as infections. In addition to bacteria the vegetable world includes parasites, such as yeasts and fungi, which are capable of producing disease. The actual production of disease depends fundamentally upon the interrelationship between the infectious agent and the invaded body. Bacteria are widely distributed in nature, but the greater number of varieties have no capacity for the production of disease. Those which produce disease are spoken of as pathogenic, and those which do not produce disease are spoken of as non-pathogenic. There are forms, however, which although they ordinarily do not produce disease, may, under certain circumstances, develop this character. Animals and plants possess certain factors of resistance to the invasion of pathogenic organisms, and the pathogenic organisms possess certain characters which favor invasion. Both animals and plants live in constant association with microorganisms, and apparently in many instances both are benefited by this association. It is well known that certain plants require for favorable development the association of the nitrifying bacteria. The intestinal canal of man, although free from

THE PRINCIPLES OF IMMUNOLOGY

Bacteria in the first few days of extra-uterine life, soon becomes inhabited by large numbers of organisms, which produce no deleterious effect under ordinary circumstances and, in fact, appear to aid in the process of digestion. Animals may adapt themselves to organisms even of the pathogenic varieties, as, for example, in the condition known as the "carrier state," in which virulent diphtheria bacilli or virulent typhoid bacilli are harbored without apparent harm. This capacity is due to certain changes which take place in the body, so that the organisms and their products do no damage. In the carrier state the organisms themselves have probably developed a state of resistance against substances produced in the host which ordinarily would destroy the organisms and neutralize their toxic products.

Parasitism.—The parasite is a living organism which carries on its existence within or upon its host, and derives its nutrition therefrom. Parasitic bacteria include both pathogenic and non-pathogenic forms. Bail has classified bacteria in three forms: (1) *Pure saprophytes*, which do not develop within living animal tissue, but derive nutrition from dead material; these may be pathogenic, provided they produce poisonous substances which may be absorbed, as is the case with the bacillus aerogenes capsulatus; (2) *pure parasites*, which live entirely within tissues, including such organisms as the anthrax bacillus; they may exist in a vegetative form for long periods of time outside the body; (3) *half parasites*, which may be pathogenic if introduced into the animal body, but do not possess the invasive character and the necessity for life within tissues exhibited by the pure parasites. Most of the bacteria pathogenic for man belong in this last group, as, for example, the bacillus typhosus and the cholera vibrio. Such organisms as these may live and grow for long periods of time in water, and in foods of various kinds, may vegetate for a certain period under unfavorable conditions, but upon introduction into a susceptible host produce local lesions and in some instances may become moderately invasive for the entire organism. Symbiosis has relatively little significance in human medicine, but certain instances occur, as, for example, the apparent symbiosis of the fusiform bacillus and the spirillum of Vincent's angina. Certain parasitic protozoa, such as the endameba histolytica of dysentery, require the associated presence of bacteria, but these latter are not necessarily pathogenic and the phenomenon is not that of symbiosis, because the endamebæ live at the expense of the bacteria, and the organisms, therefore, are not mutually advantageous to the existence of each other.

Virulence.—By the term virulence is indicated the capacity of an organism to produce disease. The degree of virulence may differ, not only between different species of organisms, but between strains within the same species. It is probably true also that individual organisms in the same culture possess different degrees of virulence. Furthermore, the virulence of a species or of a particular strain

may be altered by favorable or unfavorable conditions. Virulence, however, does not depend entirely upon characters inherent in the infectious agent, because the production of disease is an exhibition of reaction between invading organism and host. We may, therefore, say that virulence depends upon two groups of factors, those inherent in the invading organism and those dependent upon the resistance exhibited by the attacked individual. This resistance on the part of the host is represented by the condition of immunity and will be discussed subsequently. The capacity of the infecting organism in the production of disease depends upon certain inherent elements of virulence which are not well understood, upon the capacity of the organism to protect itself against the defensive mechanism of the host, upon the capacity to produce certain substances which aid invasion, and upon the development of poisonous bacterial products.

Demonstration of Virulence.—Inherent virulence of organisms may be demonstrated by the administration of accurately measured doses of the organism and observance of the effects upon susceptible animals. Ordinarily the dose is measured in the form of certain quantities of fluid culture. Growths on solid media may be measured by the use of a platinum loop so standardized as to take up approximately 2 mg. of the organisms. Such growths may also be measured by suspension in a suitable menstruum. If 5.0 c.c. of salt solution are added to a slant agar culture, fractions of the resulting 5.0 c.c. suspension contain equivalent fractions of the total surface growth. The most accurate method is that of Barber, who has developed a technic in which the use of a capillary tube permits picking a single organism out of a suspension. Of importance in considering virulence from this point of view is not only the quantity of organisms injected, but also the length of time they have lived upon artificial media, inasmuch as prolonged cultivation leads to deterioration of virulence. If a culture is maintained for a period of time without transplantation considerable numbers of the organisms die, and therefore may constitute a part of the bulk injected, at the expense of living organisms. This is not a true decrease of virulence and constitutes a factor of error. The route of injection is also of importance, because certain organisms may be virulent by one route of injection and not so by others. For example, the cholera vibrio may produce disease by introduction into the intestinal tract and is entirely without pathogenic effect when introduced subcutaneously.

The Basis of Virulence.—The studies of pathogenic bacteria have shown that they may acquire or in certain instances may lose virulence by passage through animals, and that they may lose virulence by cultivation upon artificial media. The method whereby they acquire virulence has been extensively studied. It is well known that the pneumococcus possesses a capsule when growing in animal tissue, but that it loses its capsule after artificial cultivation. This is true of certain other organisms, and it has been demonstrated that the protection afforded by the capsule makes these

organisms resistant to the defensive phenomena, phagocytosis and agglutination, as will be discussed in subsequent chapters. Therefore, capsule formation may well constitute an aid to invasion.

Aggressins.—It was found by Koch that tuberculous animals injected intraperitoneally with fresh cultures of tubercle bacilli succumb soon after the injection, and that a considerable amount of exudate appears in the peritoneum. This phenomenon seems to have been the basis of Bail's aggressin theory. Bail injected tubercle bacilli, together with sterile tuberculous exudate, into healthy guinea-pigs and found that the injected animals died in the course of twenty-four hours, while control animals inoculated with the exudate alone did not show any appreciable effect, and control animals which received tubercle bacilli alone died only after the lapse of several weeks. He argued from this that the sterile exudate must contain a substance or substances which are responsible for the increased virulence or aggressiveness of the bacilli. He named this substance "aggressin" and believed that during an infection the organisms secrete certain substances which have power to inhibit or destroy the protective powers of the host. These bodies are supposed to be formed by the living bacteria in the living body only, and the pathogenicity of bacteria is said to depend, in part at least, upon their ability to produce aggressins. Bail believed further that the germicidal activity of body fluids in natural immunity had been overemphasized. He had noted with Petterson that animals highly susceptible to anthrax often possessed sera which had marked bactericidal powers against the anthrax bacillus. If such animals were inoculated with a few hundred organisms, a number easily destroyed by their sera, they nevertheless rapidly succumbed to the disease. Bail also showed that the peritoneal fluid of guinea-pigs dying after a fatal injection of typhoid or cholera organisms possessed the ability to increase the virulence or infectivity of particular strains that would otherwise have been harmless. Experiments of this kind were also performed in dysentery, chicken cholera, pneumonia, and staphylococcus infections, and the results obtained were identical with those observed in the case of tubercle bacillus. Heating the exudate to 60° C. instead of inhibiting, increased the aggressiveness of the organisms. Small doses appeared to act relatively more strongly than larger doses. In tuberculous animals the tissues seemed to be saturated with this body, and when fluid collected in the body cavities, as happens on injection of tubercle bacilli, these fluids contained large quantities of aggressins capable of inhibiting phagocytosis by preventing the migration of polymorphonuclear leucocytes. Bail, however, was not the first to observe this particular phase of bacterial offense. Salmon and Smith, as early as 1884, noted that bacteria multiply in the tissues of their host because of a poisonous principle which is produced during their growth and multiplication. Kruse maintained that the organisms secrete ferment-like bodies (referred to as "lysins") which have

the power of inhibiting the bactericidal activity of the blood serum, thus allowing the invader opportunity for further invasion. By repeated injections of aggressin exudates into animals, Bail succeeded in immunizing these animals against various infections, thus producing *anti-aggressins*. These rendered the bacteria defenseless and permitted unhindered phagocytosis. The agglutinative power of the sera of such animals was markedly enhanced.

Wassermann and Citron, and many others, soon opposed Bail's aggressin hypothesis, by pointing out that the phenomenon can be explained without assuming that a new type of immune body is concerned. Wassermann and Citron, Wolff, Sauerbeck, and also Doerr found that the action of the so-called aggressins can be explained by the fact that exudates contain extracts of the bacteria. Artificial aggressins were prepared by making extracts of bacteria *in vitro*. It thus seems probable that the aggressins are nothing more than endotoxins which have a negative chemotatic influence and a non-specific action. Citron was able to show by means of complement-fixation that the exudates contain free bacterial receptors, which by absorbing immune bodies, tend to neutralize the destructive power of these antibodies. Levy and Fornet showed that fresh twenty-four- to forty-eight-hour culture filtrates of bacillus typhosus, paratyphosus, pyocyaneus and proteus possess non-specific aggressive powers and, according to Ikomikoff, aggressins of bacillus coli, staphylococci, and vibrios will act interchangeably, thus showing the non-specific nature of these substances. From Zinsser and Dwyer's experiments these bodies appear to be practically identical with anaphylatoxins (see page 218). The addition of anaphylatoxin to bacteria will change a sublethal dose into a lethal dose.

Closely related to the aggressins are the "virulins" of Rosenow. This author found that freshly isolated cultures of pneumococci were not readily phagocytized, but this property was lost on repeated subculture. He prepared salt solution extracts of the virulent strains. Upon treating avirulent strains for twenty-four hours or more with these extracts, the avirulent organisms became virulent for animals, and at the same time resistant to phagocytosis. The substance contained in the salt solution extracts capable of rendering the organisms virulent was named virulin. This substance appears to be essentially the same as the aggressin prepared *in vitro* by Wassermann and Citron. In our opinion, these extracts, whether prepared in the form of exudates or as extracts, contain poisonous bodies which augment the invasiveness of the organism. They may be non-specific bacterial proteins or protein products, such as are probably contained in so-called anaphylatoxin. They may be in part endotoxins. The anti-aggressins of Bail are agglutinative and are probably called forth by the injection of the extracted bacterial proteins in the exudates or extracts. It seems probable also that the effect of these anti-aggressins may depend upon their agglutinative capacity. The subject is confused and in-

tricate, and whilst at present we are disposed to regard the aggressins as extracts of the bacterial proteins and their split products, as well, perhaps, as exotoxic in nature, further study may offer more complete and satisfactory explanation of the problem.

Production of Poisonous Substances.—The virulence of organisms depends, to a certain extent, upon the poisonous substances which they produce. Nevertheless, virulence is not necessarily parallel to the capacity for production of these toxic substances. The poisonous bacterial products may be divided into four groups, namely, the *ptomains*, which are the result of decomposition of the media upon which the bacteria grow; the *exotoxins* or true toxins, which are soluble poisons produced by the life activities of the bacteria and easily absorbed and diffused in the body of the host; the *endotoxins*, which develop within the bodies of the bacteria and are liberated probably only upon the death and disintegration of the bacteria; and *poisonous bacterial proteins*, which result in large part from the breaking down of the protein molecules which go to constitute the bacterial substance.

The *ptomains* are formed from the decomposition of the media upon which bacteria grow, provided these media are nitrogenous in nature. The ptomains are basic substances formed not from the bacteria themselves, but from the decomposition products of those media which contain nitrogenous material, especially proteins whose nitrogen is in the form of amino-acids. Most ptomains are combinations simply of carbon, hydrogen, and nitrogen, and they may be divided into three groups, the first of which includes methylamine, dimethylamine, and trimethylamine; the second group somewhat more complex, contains putrescin and cadavarin; the third group, the so-called cholin group, contains, in addition to cholin, neurin, muscarin, and betain. The cholin group are derivatives of lecithin. Cholin itself is found in extremely minute amounts in body cells and has a relatively low degree of toxicity. It is a substance which has been the subject of much experiment and hypothesis, but there is no very good reason for believing that it has any great pathologic importance. Neurin may be transformed from cholin, and although somewhat similar chemically, it is highly poisonous. Muscarin is a crystalline alkaloid obtained from poisonous mushrooms, but is also formed by the decomposition of fish; its chemical composition is very closely similar to that of neurin, and it may be prepared synthetically from cholin. Both neurin and muscarin produce definite toxic symptoms in man following subcutaneous injection of 1 to 3 milligrams, but when given by mouth approximately ten times this amount are required, indicating that probably the liver breaks up and detoxifies that which is absorbed from the intestine. Betain is a constituent of plant tissues and has a toxicity from one-tenth to one-twentieth that of neurin and muscarin. The simpler ptomains are not extremely toxic. The ptomains as a group are not specific in any sense, except in so far as they are dependent on the chemical

composition of the media upon which the bacteria grow, and any differences in constitution of ptomains are differences due to variations in medium rather than variations of bacteria. In this respect they differ from toxins. Furthermore, it is not possible to produce immune substances against ptomains. Ptomains are not to be confused with toxins produced by bacillus botulinus, by bacillus enteritidis, or other members of the "food-poisoning" group, which are true toxins and are capable of inducing the formation of antitoxins. Food poisoning may, therefore, be due to the decomposition of food with the production of ptomains which are absorbed and produce toxic symptoms, or may be due to the presence in food of toxins produced by the bacillus botulinus and similar organisms. In addition to the ptomains which contain C, H and N, a fourth group contains also oxygen, as exemplified in the substance sepsin obtained from decomposing yeast cells. This is closely related to cadavarin in its chemical composition and acts as a powerful dilator of intestinal capillary blood-vessels from which diapedesis may occur.

The true toxins or exotoxins are soluble and diffusible poisonous substances produced by the life activity of bacteria. They may be produced when the organisms exist in a parasitic state or when they grow upon artificial media and the nature of a toxin for any given species is not determined by the medium upon which the organisms grow, except in so far as certain media favor the production of greater amounts of toxin than do others. The diphtheria bacillus produces the same toxin regardless of the medium upon which it is grown, although nutrient veal broth is the most favorable for toxin formation. The same general statement is true of the tetanus bacillus and those other organisms which produce toxins. Toxins are unlike ptomains in that they have not a definite chemical composition and in that they serve to induce antitoxin formation. They have certain resemblances to enzymes, but are probably not identical with enzymes. The nature of toxins, their action, and other details are considered in the chapter on toxins and antitoxins.

The endotoxins develop within the bodies of bacteria and are not secreted into the surrounding medium. They apparently are only liberated upon the death and disintegration of the organisms. It is not certain that they can be differentiated absolutely from the poisonous bacterial proteins, and it is extremely difficult to induce antitoxin formation by their use. If they are injected into an animal the animal may produce agglutinins and precipitins, but not antitoxin. This subject also is discussed subsequently.

Poisonous Bacterial Proteins.—The whole protein of certain bacteria is poisonous, and the work of Vaughan and Novy shows that the split products of bacterial proteins produced by treatment with alkalinized alcohol are extremely toxic. These substances apparently are not specific as regards the bacteria from which they originate, but owing to their poisonous properties they may add to the virulence of the organisms. Similarly toxic split products may

be obtained from other proteins, such as those of cheese and milk. The poisonous effect is in some way connected with the foreign character of proteins. In some respects these substances resemble ptomains, but they are certainly not of the same constitution. They are obtained from bacteria regardless of whether these produce toxins, endotoxins, or ptomains, and are fatal for animals in very short periods of time. The methods for the production of endotoxins are such as may lead to splitting of bacterial proteins, and at the present time no satisfactory differentiation can be made. The chapter on anaphylaxis and hypersusceptibility will present a discussion of the poisonous substance called anaphylatoxin, which may also be related to the general group of poisonous split products. The influence of toxin on invasion by certain bacteria is illustrated by the recent work of Bullock and Cramer. They found that *bacillus aerogenes capsulatus*, *vibrio septique*, *bacillus edematiens*, and often *bacillus tetani*, when completely freed of toxin by washing or by heating to 80° C. for one-half hour do not produce the special disease upon injection into the rat or guinea-pig. The usual defenses of the animal, such as bacteriolysis and phagocytosis, are sufficient to rid it of the bacteria in the absence of toxins. A point of further interest in this work is the discovery that if a small dose of a soluble ionizable calcium salt be injected before or at the same time as the spores or toxin-free bacteria, the defenses are broken down and the special disease results. The experiments showed that this is not the result of action upon the bacteria, but is due rather to some influence upon the host. Bullock and Cramer suggest the name "cataphylaxis" for the rupture of defense. Other salts have no such effect, and it is possible to demonstrate the antagonistic action of magnesium upon calcium in similar experiments. It is difficult to find a series of experiments showing more clearly the delicacy of balance between resistance and infection.

Alterations of Virulence—Increase of Virulence.—As has been indicated above, virulence may be increased by the passage of organisms through animals, and this method is commonly employed in laboratory work. The increase of virulence of the pneumococcus by passage through mice is an excellent example of the process. The organisms are injected intraperitoneally, recovered upon the death of the animal, cultivated for twenty-four hours, reinoculated, and the process repeated until a satisfactory degree of virulence is obtained. The degree of virulence is usually measured in terms of the bulk of broth culture which will kill an animal in a given period of time. In the case of some bacteria an increase of virulence by animal passage is only effective for the animal concerned; and the fact that an organism exhibits increased virulence for a guinea-pig does not necessarily presuppose that the same increase will apply to other animals. Not only is this true of direct animal passage but, as has been shown by Danysz, cultivation of an organism upon media containing rat tissue may increase the virulence for the rat but not for

other animals. The importance of proper selection of the animal species for increasing bacterial virulence is emphasized by the work of Hussy, who found that the passage of streptococci through mammals and fish increased the virulence, but their passage through birds decreased the virulence. In the increase of virulence by means of animal passage, the organism apparently may develop a mechanism of resistance against the protective activity of the animal body, as has been discussed above. Another method of increasing virulence is to place the organism in collodion sacks. These are planted in the peritoneal cavity of an animal and apparently the slow diffusion of the animal fluids into the sack permits the organism to acquire resistance to the antagonistic substances of the animal and thus increases its virulence. A third method of increasing virulence is to grow organisms upon media which contain blood serum or other animal fluids. By several transfers upon such media the organisms may acquire resistance similar to that obtained in the other methods. A fourth method has been applied, depending upon the separation of the more virulent individuals in a culture from the less virulent at the height of phagocytosis. A culture is inoculated into the peritoneal cavity of an animal, such as the guinea-pig, and by removing small quantities at regular intervals the time of greatest phagocytosis by the peritoneal cells is determined. The entire exudate is then withdrawn and slowly centrifuged, so as to throw down the cells, leaving the unphagocyted bacteria in the supernatant fluid. The organisms in the supernatant fluids are cultivated, and if they are not sufficiently virulent the process may be repeated until a satisfactory culture is obtained.

Decrease of Virulence.—The virulence of pathogenic organisms may be decreased by removing them from the favorable environment of the animal host and growing them upon artificial culture media. As they become accustomed to this type of existence they usually lose considerably in virulence. As has been indicated above, there are instances where animal passage may decrease the virulence of certain infective agents. Whereas the virus of rabies increases up to a standard maximum on passage through rabbits, similar passage through monkeys will decrease its virulence. It is probable, also, that the natural passage from dog to dog decreases virulence. It is now generally accepted that cowpox is the same disease as smallpox, yet the inoculation of cowpox into man produces a very mild form of disease. Therefore, it is to be presumed that the passage of smallpox virus through the calf reduces the virulence. A similar example is found in the work of Hussy on the streptococcus quoted above. Decrease of virulence by animal passage is not clearly understood. It may be due to the same factors that influence virulence in artificial culture media, whereby the organisms in an unfavorable environment lose their ability to combat the resistance of the animal host, or it may be due to a direct lowering of virulence as the result of more or less successful attacks of the pro-

tective mechanism of the animal body. The influence of heat on the virulence of organisms is now well known. The degree of heat and the time of exposure must be so adjusted as to reduce virulence without causing actual death of the organisms. Similar reduction of virulence or attenuation may be accomplished by growing the organisms at temperatures which are not optimal. The first example of this was Pasteur's work in the attenuation of anthrax cultures by growth at 42° to 43° C. The attenuation by means of drying was practised in the classical work of Pasteur on rabies. The virus contained in the spinal cord of rabbits was subjected to desiccation, and it was found that the longer the time of desiccation the less potent was the virus. Chemical agents, such as phenol, acids, iodine and its salts, potassium bichromate, and others may also be used in proper concentrations and for proper periods of time to produce attenuation. Physical agencies, such as growth under pressure, the influence of light, etc., have been employed for purposes of attenuation. Of interest in connection with attenuation is the fact that certain organisms, when introduced into the body, vary in virulence, depending upon the route of introduction. For example, the virus of rabies may be injected intravenously into goats and sheep without producing rabies. This injection, however, serves to confer a certain degree of immunity upon the animals. As has been mentioned before, the organism of cholera may be injected subcutaneously without producing disease, and within certain limitations aids in the protection against invasion by these organisms through the intestinal canal.

CHAPTER II

GENERAL CONDITIONS OF INFECTION AND RESISTANCE

THE PRODUCTION OF INFECTIONS.

ENTRANCE OF THE INVADING ORGANISM.

TYPES OF INFECTIOUS DISEASES.

FACTORS FAVORING THE INVADER.

FACTORS INHIBITING THE INVADER.

FACTORS OPERATING AGAINST RESISTANCE OF HOST.

FACTORS FAVORING THE HOST.

THE COURSE OF ACUTE INFECTIOUS DISEASE.

The Production of Infection.—The widespread dissemination of bacteria in nature is such that they have ready access to plants and animals. Invasion by pathogenic forms may set up infection. Whether or not the infection may lead to disease depends upon the final relationship established between the invader and the invaded body. There is probably no condition under which animals or plants fail to exhibit some degree of resistance to the invading organism, and similarly the latter attempts to accommodate itself to the conditions found in the invaded host. If the resistance be not sufficient to overcome the invader, infection results. The production of disease, however, depends upon the superior powers of the invader over the resistance of the host. Occasionally a mutual adaptation appears, under which circumstances an animal may be infected by an organism, but shows no symptom or sign of disease. Not infrequently the trypanosoma Lewisi is found in the blood stream of rats, the rats continuing to live an apparently normal existence. A similar mutual adaptation is found in the "carrier state," wherein man may harbor virulent diphtheria bacilli or other organisms without any evidence of disease. Mutual adaptation is not attained without a struggle on the part of both invader and host, and infectious disease results when the invading organism triumphs. This does not mean permanence of infection, because even although disease is established, the defenses of the host continue to operate, and often are augmented in such a way that ultimately the infection disappears. This accounts for the self-limitation of most of the acute infectious diseases. The increase in defensive powers may in certain diseases become permanent and immunity thereby be established. In all cases of recovery from acute infections immunity of some duration appears, although it may be limited to a few weeks or a few months.

Entrance of the Invader.—The entrance of the invading organism may be due to an interruption of continuity of those surfaces of the body which ordinarily are impermeable to bacterial invasion. These surfaces include skin and the mucous membranes of the respiratory, alimentary, and genito-urinary tracts. The interruption

of continuity may be due to trauma or may result from profuse growth of bacteria on the surface with the elaboration of poisonous products which may kill the epithelial cells. The former condition is exemplified in infected wounds and the latter in infection by diphtheria bacilli, streptococci, and fungi, such as produce favus, thrush, and pityriasis. Entrance may be favored by changes in the character of secretions, as, for example, the reduction of acidity of the gastric juice in certain forms of chronic gastritis. The bacteria may be implanted in some site which favors their multiplication, as, for example, in the crypts of the tonsils, in the crevices between unclean teeth and in hair follicles. Multiplication in these situations favors the production of poisonous products which may by destruction of cells serve to interrupt surface continuity. Somewhat similar is the fact that extensive destruction of tissues may provide dead material in which saprophytes may develop, and if this material is so deep as to be excluded from the access of air, conditions favorable to the development of anaërobes are produced. Infection may be favored by the movement of cells and fluids. For example, although leucocytes may take up bacteria, they do not invariably destroy them, and the migration of such leucocytes may lead to the dissemination of organisms by the subsequent death of the leucocyte. The movement of lymph may favor invasion as is seen not uncommonly in those cases of infections of the hand by streptococcus, wherein the lymph flow carries the organisms so as to set up infections of the lymph-vessels and the lymph-nodes, and even of the blood stream. Gaining access to the blood, the circulation of this fluid tissue may deposit bacteria in numerous foci throughout the body. The route of invasion depends somewhat upon the type of organism, those of typhoid fever, dysentery, and cholera, gaining access to the intestinal canal through the mouth. Their implantation upon the skin is of no significance, except that they may thence be transferred to the mouth. The gonococcus produces no lesions of the intestinal canal, but implanted in the genital tract, the eye, or the endocardium leads to serious results. If plague bacilli be inoculated subcutaneously in rats a large percentage of the animals survive, but if implanted in the lower respiratory tract small doses suffice to produce fatal infections. The pneumococcus appears to infect man only through the respiratory tract. This phenomenon probably depends in part upon a local susceptibility to the organisms.

Types of Infectious Disease.—The types of infectious disease are differentiated according to the method of invasion and dissemination. An organism may grow locally and produce only local manifestations, as seen in a small abscess. It may grow locally and produce marked general disturbances, as is the case in diphtheria, in which instance, although organisms may enter the blood stream, they are usually confined to some focus, such as the tonsils. They elaborate in that situation poisonous substances which are absorbed and set up general manifestations of intoxication. Certain other

diseases may produce marked local manifestations and rapidly invade the blood stream, as is true of typhoid fever. This organism enters the lymph-nodes of the intestinal tract, produces enlargement, softening, and necrosis. The diarrhoea in these cases is largely if not wholly due to the local lesions, but the severe general manifestations are due principally to the entrance of the organisms into the blood stream. Other diseases may show little local manifestation, as is true of tetanus, but even with slight local disturbances profound general symptoms occur as the result of absorption of toxin. Other diseases, such as anthrax, may show little local manifestation, but rapidly exhibit generalized infection through the blood stream. *Infection* then simply signifies successful invasion. *Bacteremia* signifies the presence of organisms in the blood. *Septicemia* signifies blood infection associated with the production of toxic substances. *Pyemia* indicates that bacteria are present in the blood stream and because of lodgment in numerous situations produce multiple abscesses. *Sapremia* indicates absorption of toxic products from the growth of saprophytic organisms. *Primary infections* are those which occur without any decrease of resistance due to another infection. *Secondary infections* occur in individuals already suffering from an infection of another nature. Such an infection is well exemplified in the secondary infection of a tuberculous cavity of the lung by staphylococcus. *Terminal infections* are those which occur near the fatal termination of some other disease, whether that other disease be of bacterial nature or of some other origin. Infections of this type are seen in the terminal broncho-pneumonias and septicemias which occur in the course of certain chronic diseases. *Mixed or multiple infections* are not rare and it is sometimes difficult to determine which infection is of greater importance. There is no doubt that one infection influences another existing at the same time and usually in a manner deleterious to the patient. Infection with measles or lobar pneumonia may excite latent tuberculosis into activity. Duke reports the lighting up of latent syphilis by an attack of typhoid fever and of latent gonorrhea by an attack of tonsillitis. The removal of one chronic infection may favorably influence another, as seen in the relief of certain cases of pyorrhea alveolaris by the removal of infected tonsils and in numerous other instances of multiple chronic infections.

Factors Favoring the Invader.—The small size of pathogenic bacteria and protozoa aids in their avoidance of detection, favors transportation, and aids in penetration. The rapidity of multiplication of such organisms is of considerable importance to their pathogenic powers. Those bacteria which form spores resist destructive agents and can resume activity when favorable conditions present. Certain of the protozoa, more particularly the endamebæ, are capable of forming cysts which are more resistant to unfavorable environment than the active organism. Either in the active state or in the vegetative state, organisms may persist for a long time in the so-called carriers, in intermediate hosts, or living as saprophytes.

The microparasites, therefore, can be said to have a ready adaptability to varying environment and to be aided in propagation by their ability to derive nutrition from food-stuffs which possess wide differences in constitution. Certain bacteria apparently can produce their own protein from amino-acids and have no difficulty in deriving nutrition from whole proteins. As has previously been indicated, those factors which go to increase virulence of organisms, such as capsule formation and the production of toxic substances, aid materially in invasion.


Factors Inhibiting the Invader.—Although rapid multiplication aids invasion, nevertheless, the brief life period which most of the microparasites exhibit is an influence operating against rather than in favor of invasion. Many pathogenic organisms are susceptible to the destructive influence of light, heat, desiccation, etc. In certain instances the life of organisms outside an animal body operates to reduce virulence and therefore to inhibit the capacity for invasion and production of disease.

Factors Operating Against Resistance.—The animal host is subjected to the attacks of invading organisms because of the multiplicity of contacts with the environment. The large body surface and locomotion of the body are influences favoring approximation of the invader. Certain living activities, such as the ingestion of foods and water, coitus, and the ready availability of superficial orifices, such as the nose, ears, mouth, anus, genital orifices, all aid invasion. The fact that most animals have a constant body temperature and that their tissues are continually moist, provides conditions favorable to the invading organism. Although light rays beyond the violet end of the spectrum have a certain capacity for the penetration of tissues, yet ordinary sunlight exhibits very little penetrability; therefore, the construction of the body is such that the inhibitory effect of light is not brought to bear upon organisms that have already gained entrance. The anatomy of the body provides certain structures which are relatively inactive, such as the appendix vermiformis and the crypts of the tonsils where organisms find moisture, warmth, and darkness, suitable for their development. In chronic infections, particularly by the tubercle bacillus, necrotic tissues, or actual cavities may exist in contact with surfaces and with the outer air, and both conditions operate to reduce resistance by providing favorable places for bacterial multiplication. The circulation of lymph and blood may operate against the host if organisms are particularly virulent. Inspiration of contaminated air may also serve to aid invaders. The resistance of the host may, in a manner as yet unexplained, be decreased in general by bodily fatigue, exposure to heat and cold, poor hygienic surroundings, deleterious gases, or improper diet. The extremes of life, childhood and age, are associated with reduced resistance. Drugs, operative procedures, improper diet, and similar conditions favor infection.

Factors Favoring the Host.—The possession of intelligence by

the higher forms of animal life aids in the detection and elimination of infective organisms. Not only may this be accomplished by voluntary movement, but the purposeful action of involuntary reflexes may similarly aid the host. The body possesses a variety of defenses in the form of structure, secretions, chemical substances, cellular activity, all of which serve to aid in its protection in connection with natural and acquired resistance to disease. These will be discussed in the next chapter. Plants produce certain diastases, aromatic products, aldehydes, and other substances which create in the plant a state deleterious to germination of harmful invaders. Pigments such as chlorophyll may destroy toxic substances and even bacteria in a manner somewhat similar to the action of bile pigment.

The Course of Infectious Disease.—The exact moment of invasion of an infectious agent is difficult to determine, but in cases of infectious disease, the time of exposure to infection can usually be stated to have occurred within the limits of a few hours. Following the moment of invasion there occurs a period of incubation during which the host exhibits no symptom of infection. This period of incubation in some diseases is extremely variable, whereas in others it is relatively fixed. In diphtheria incubation may apparently vary from twenty-four hours up to nine or ten days, and certain other diseases show similar variation. In scarlet fever, on the other hand, the incubation period is very commonly five days, and numerous other diseases show similar fixity of incubation time. Following the period of incubation the less violent infectious diseases show a short period of prodromal symptoms in which headache, malaise, and other minor manifestations may appear. The next period, that of onset of disease or so-called invasion, may be frank or insidious. Lobar pneumonia may develop within a period of a few hours and exemplifies frank onset. As a contrast, typhoid fever is likely to occupy a week or ten days between the period of prodromal symptoms and the full development of disease, thus illustrating insidious onset. That period during which the disease is at its height is called the fastigium or acme. Following the fastigium comes the period of decline or defervescence. This may be by crisis or lysis. Crisis is seen in approximately half the cases of lobar pneumonia, in which the decline occurs in a period of a few hours. Defervescence by lysis is seen in a large number of infectious diseases and is particularly well exemplified by typhoid fever in which several days, a week, or more, may be consumed. Convalescence indicates that period during which the symptoms of disease have practically disappeared and the patient gradually recovers and is restored to normal. At any period the infection may become so overwhelming as to cause the death of the individual. Chronic infectious diseases exhibit no such regularity of development and decline. In contrast to the acute infections, these are not likely to be self-limited, but progress until they have reached a point of such great severity, or of such complete exhaustion of the host that death ensues.



CHAPTER III

THE GENERAL PHENOMENA OF IMMUNITY

TYPES OF IMMUNITY.
 NATURAL IMMUNITY.
 SPECIES.
 RACIAL.
 FAMILY.
 INDIVIDUAL.
 INHERITED IMMUNITY.
 ACQUIRED IMMUNITY.
 NATURALLY ACQUIRED.
 ARTIFICIALLY ACQUIRED.
 ACTIVE ARTIFICIALLY ACQUIRED.
 INOCULATION OF LIVING VIRUS IN HEALTH.
 USE OF ATTENUATED VIRUS.
 USE OF DEAD BACTERIA.
 USE OF BACTERIAL PRODUCTS.
 PASSIVE ARTIFICIALLY ACQUIRED.
THEORIES OF THE NATURE OF IMMUNITY.
 THE EHRLICH SIDE-CHAIN THEORY.
 THE EHRLICH CLASSIFICATION OF IMMUNE BODIES.
 CRITICISM OF THE EHRLICH HYPOTHESIS.
THE SPECIFICITY OF IMMUNE REACTIONS.
 NON-SPECIFIC THERAPY OF INFECTIOUS DISEASES.
THE SITE OF ANTIBODY FORMATION.
 PRODUCTION OF ANTIBODIES AT SITE OF INJECTION.

Types of Immunity.—Resistance to disease may be natural or acquired. If natural it may be of a species, race, family, or individual character. If acquired it may be naturally acquired, as seen in the immunity following an attack of infectious disease, or it may be artificially acquired. If artificially acquired it may be the result of active immunization or of passive immunization. Artificially acquired active immunity is such as may follow the injection of various antigens, such as toxins, bacteria, and numerous other substances. Artificially acquired passive immunity is the result of transfer of active immunity from an immune animal to a normal animal, which latter becomes passively immunized.

Natural Immunity.—Although the term immunity may be considered as equivalent to the capacity for resisting disease, nevertheless, in common usage it often implies an increase of resistance. In estimating an increase of resistance a normal degree must be presupposed and the determination of the normal is extremely difficult. In considering natural immunity the term is used in contrast to susceptibility and is not comparable to a normal level of resistance. Natural resistance to disease is favored by structure, movement, fluids, and secretions of the body. Structurally the skin is practically impermeable to bacteria. In a general way this is true of mucous membranes, although we know that certain organisms may pass through mucous membranes of the intestinal tract without any

lesions of the surface. Crypt-like structures, such as hair follicles, sweat glands, crypts of the tonsils, gastro-intestinal glands, urethral glands, may serve as foci where bacteria are able to multiply, and may thus determine penetration by the organism. Accessory structures of the skin, such as the hairs of the anterior nares and the cilia of certain parts of the respiratory tract, aid in either filtering the air or in propelling lodged organisms toward external orifices. The nature of certain secretions may be antagonistic to the growth of certain bacteria either by virtue of chemical substances, such as the hydrochloric acid of the gastric juice, normal alkali of the saliva and upper intestinal tract, or by virtue of digestive ferments which may act deleteriously upon bacterial growth. The movement of secretions, as, for example, that of the conjunctival sac, may favor the elimination of organisms. Bodily movement is of considerable value in resistance to infection, whether it be the simple process of wiping away irritating substances or the more intricate process of bathing either with water or with definite anti-bacterial fluids. Reflexes such as coughing, sneezing, and vomiting are definitely purposive in protection. The movement of materials in the intestinal canal serves to prevent any too great bacterial activity, and if in spite of normal intestinal movement irritative substances are formed, the response by diarrhea serves a useful purpose in elimination. Internally the fluids of the body, more particularly the blood, contain definite anti-bacterial and anti-infective substances. In addition to these the non-specific ferments of the body fluids aid in combating infection. The acidity or alkalinity of fluids within the body, as well as certain substances of unknown nature, may serve to retard or prevent bacterial invasion. Of great importance in protection is the reaction of inflammation. In the course of this process fluids and cells are exuded from the vessels. The exudation of fluids upon surfaces aids in washing away bacteria, as, for example, the profuse exudation of fluid in acute coryza and acute enteritis. Accumulation of fluids may serve to dilute bacterial poisons and by diffusion and absorption aid in the elimination of these poisons. The cells which form part of the exudate possess, as characteristic functions, the capacity of taking up bacteria by phagocytosis and destroying them. The formation of fibrin in the exudate, as well as the subsequent proliferation of fixed tissue cells, serves to delimit the process and thereby aid in the prevention of widespread dissemination of the organisms. In superficial inflammations the exfoliation of diseased cells, as in scarlatina, may aid in the elimination of the infective virus. This does not mean, however, that such cells retain an infective character after long periods of desiccation.

The physiological activity of cells in the body may be so excited as to aid in the elimination of toxic products, as exemplified by the early increase of activity in infectious disease. If the toxic material be sufficiently virulent this period of hyperactivity may be suc-

ceeded by one of depression. The stimulation of cells in the production of antitoxic and anti-bacterial substances will be discussed subsequently.

Classification of Natural Immunity—*Species Immunity*.—As has been indicated above, natural immunity may be found in species, races, families, or individuals. It is profitable to emphasize again that what we speak of as species immunity expresses a difference in susceptibility exhibited by certain species as contrasted with others. Whereas man is susceptible to such diseases as syphilis, gonorrhea, cholera, and diphtheria, numerous other species are resistant to these diseases. It is possible to inoculate syphilis in higher apes, in the rabbit, possibly in the guinea-pig and other animals, but even successful inoculation shows a greater degree of resistance than is possessed by man. Conversely, man is not susceptible to hog-cholera, chicken-cholera, rat-typhoid, and certain other diseases. Man is susceptible to the bacillus of human tuberculosis, but less so to that of bovine tuberculosis, still less to that of avian tuberculosis, and not at all to that of fish tuberculosis. In fact, with the exception of the rabbit, fish tuberculosis is not transferable to any of the warm-blooded animals. Fish are not susceptible to human tuberculosis. Practically all animals are susceptible to snake venoms except the hog. Man is highly susceptible to pneumococcus and to bacillus pestis, but fowl are resistant to both these organisms. Metchnikoff showed that certain species of insects are susceptible to diphtheria toxin whilst others are not. Man is susceptible to trypanosoma gambiense, but is resistant to trypanosoma naganæ. In some instances these variations in susceptibility and resistance depend upon the environment. For example, frogs kept in low temperature are not susceptible to anthrax, but if kept in a temperature of 35° C. they succumb to the disease. Similarly it was found that if lizards are kept at 16° C. they could not be infected with plague, but at a higher temperature were susceptible. The work of Pasteur with anthrax in fowl is a classical experiment. He found that if he kept fowl at low temperatures they became susceptible to anthrax because of the decrease of body temperature; but if they were allowed to maintain their normally high body temperature they were resistant. The temperature of most of the lower mammalia is higher than that of man, but the difference is not sufficiently great to explain all the variations in susceptibility and resistance.

Racial immunity probably exists but cannot be so conclusively proven in man as is true of species immunity. It is generally believed that Caucasians are less susceptible to tuberculosis than negroes. That this is an inherent character of the race appears to be somewhat doubtful. Difference in hygienic conditions and in degree of exposure to the disease may account for much that appears to be racial susceptibility. It is possible that the superior hygienic conditions of whites in northern latitudes explains this difference. It is also possible that having been the victims of tuberculosis for

many centuries a certain degree of racial immunity has been established by virtue of the elimination of more susceptible individuals and the survival of the more resistant. It is apparently true that when an infectious disease first attacks a race, it is more virulent than in those races where it is commonly found. The native African when brought into contact with tuberculosis appears to be attacked violently. The decimation of the population of Iceland after the introduction of measles was one of the horrors of improved communications; subsequent epidemics of the disease in the same people have been considerably less fatal. The introduction of syphilis into the American Indian showed a virulence unknown among the Caucasians. Smallpox materially aided the Spaniard in his conquest of Mexico. The negro is supposed to be less susceptible to yellow fever than is the Caucasian, but careful investigation would make it appear that in infancy and childhood acquired immunity is established by mild attacks of the disease. The recent work of Love and Davenport shows that among 500,000 troops illness was 19 per cent. more frequent among negro than among white troops. The negro was apparently less resistant to pneumonia, tuberculosis, and smallpox than the white. The negro was more resistant to skin diseases, but contracted venereal disease readily and suffered more than the whites from extension and complications of venereal disease. Borell has reported that the Senegalese are very susceptible to pneumonia even in their own country. On transportation to France during the World War more than 5 per cent. succumbed to pneumonia before they had become acclimated, but in those who had been in France two or three years, the death-rate from pneumonia was much reduced; only 2 in 7000 troops died of pneumonia. Whether this reduction is due to acclimatization or the early elimination of the more susceptible is an open question. An apparent racial immunity to malaria may be explained by the persistence of this disease for many years following a childhood infection. In Australia, New Zealand, and Tasmania during the years 1906-1908 there were only about half the deaths per thousand inhabitants as the result of tuberculosis than occurred in Ireland, Norway, and Japan, during the same period; whilst the rate decreased regularly in the former countries it increased in the latter. This appears to favor the idea of racial differences of susceptibility, but a careful analysis of all the conditions may show that climate, mode of life, and hygienic conditions have a considerable influence. In the lower animals racial difference may be more satisfactorily illustrated. Common sheep are susceptible to anthrax, whereas the Algerian sheep seem to be immune. The culex mosquito rarely harbors the malarial parasite, whereas the anopheles are commonly infected. The field mouse is highly susceptible to glanders, whilst the white mouse is immune. The gray mouse is more resistant to streptococcus infections than is the white mouse. The common rat is more resistant to anthrax than is the white rat.


Family Immunity.—Members of certain families may through generations appear to be especially susceptible to such diseases as tuberculosis and rheumatism or the converse may be true. In the case of tuberculosis this difference may be the result of conformation of the body. The physical character of flat, narrow chest and thin skin apparently go hand in hand with susceptibility to tuberculosis, whereas the well-rounded chest appears to indicate resistance. In a family with whose history we are familiar the blondes have almost invariably succumbed to tuberculosis and the brunettes living under the same conditions and in intimate association have been resistant. This must be due to inherent constitutional characters and is not to be considered as a difference due to complexion alone.

Individual Immunity.—Variations of individual resistance or immunity are seen frequently. It is true that the extremes of age show a certain proneness to infection and that this varies somewhat with individuals. Excellent examples of individual resistance are seen in great epidemics where some of those exposed apparently in the same manner and under the same hygienic conditions as others show either complete resistance to the disease, or, if they are attacked, develop only moderate or slight attacks. Infected water and foods consumed by a population may lead to disease in only a small portion of those exposed. Individual variations in animals are very frequent and offer a considerable source of error in the interpretation of experimental results. If a series of guinea-pigs be injected with the same dose of anthrax bacilli, all will die at practically the same time, but if rabbits be treated in the same way some die within two days, others die subsequently, and still others are completely resistant. On the other hand, rabbits are all susceptible to chicken-cholera, whereas the guinea-pig shows great individual difference. Although a large number of children suffer from tonsillar infections, yet the incidence of acute articular rheumatism or of endocarditis is small and variable. Instances might be multiplied indefinitely of individual variations in resistance, but the phenomenon is one of common knowledge.

Inherited Immunity.—The immunity transferred from parent to offspring may be a natural immunity or an immunity acquired by the parent. The transfer of natural immunity may be seen in racial, species, and family manifestations, and is probably a true transfer through the germ plasm. Congenital immunity may arise either in the form of an active immunity developed in the fetus because of the presence of antigens in the circulating blood of the mother, or may be in the form of passive immunity transferred from the blood of the mother to that of the fetus. It is conceivable that the fetus may survive an attack of disease transmitted from the mother and thereby become immune. It has been known since the time of Pasteur that certain dogs are immune to rabies. Remlinger has found that the guinea-pig may transfer rabies to the fetus and puppies have been known to become rabid several months after

birth without any evidence of having been bitten, the disease therefore probably having been contracted *in utero*. Immunity in dogs may be explained by direct transmission of immunity from the mother, or by survival of the disease in uterine or early post-uterine life.

Acquired Immunity—*Naturally Acquired Immunity.*—The acquisition of immunity may be through so-called natural processes, such as passing through and recovering from an infectious disease, or it may be induced and artificially acquired by special methods of immunization to be described. In both these instances, although the normal level of resistance cannot always be accurately determined, yet there is no doubt that the acquired immunity represents a higher level of resistance than is normally possessed. For example, the fact that when a patient has survived an attack of such a disease as scarlatina and then in spite of repeated and intimate exposure resists infection, leaves no doubt that his acquired immunity represents a higher level of resistance than he possessed before the attack of the disease. The diseases which confer a lasting immunity include acute anterior poliomyelitis, chickenpox, cholera, epidemic cerebrospinal meningitis, measles, mumps, plague, scarlatina, smallpox, typhoid fever, typhus fever, whooping-cough, and yellow fever. The question as to whether or not syphilis confers a lasting immunity has been reopened by the discovery of the Wassermann test and by the work of Warthin. The Wassermann test has shown that many cases of apparently cured syphilis are really in a latent stage of the disease. Warthin has found the *treponema pallidum* in various organs at autopsy on syphilitics who clinically appeared to be free from the disease. If Warthin's work can be confirmed in a large number of cases it would appear that syphilitic infection remains latent throughout the life of the individual in the vast majority of cases, even in spite of the fact that the Wassermann test is negative and no clinical signs of the disease are demonstrable. If syphilis be curable, the reported occurrence of second infections in a small number of instances would make it appear that any immunity which may develop is not permanent. The long duration of the disease would account for the small number of reinfections reported. Immunity in tuberculosis has been extensively studied, and as yet no final and conclusive statements can be made. It seems probable that tuberculosis is never completely eliminated from the body, and although the patient exhibits no symptom nor sign, he still may harbor the disease. The studies of Opie and others would make it appear that the development of tuberculosis in adult life is traceable directly to old lesions which occurred in childhood. The fact that a very large number of individuals show at autopsy small lesions indicates the prevalence of the disease. Subsequent active development, following encapsulation of a lesion, appears to be due to certain factors which either reduce the protective properties of the body or excite the organisms to renewed activity, or both.



Artificially Acquired Immunity.—The artificial acquisition of immunity may be the result of active development of immune substances in the organism or it may be due to the transfer into the organism of immune substances from an immune animal. Artificially acquired immunity differs from naturally acquired immunity in that it is likely to be less durable. If acquired by active immunization the duration is likely to be considerably greater than if acquired by passive immunization. In the discussion of immunity it is well to keep clearly in mind the definition of antigen and antibody. The antigen is a substance which upon introduction into the body in proper amounts and under suitable conditions induces the formation of a special antagonistic substance, the antibody. Conversely the antibody is the substance produced as a result of the introduction of antigen. Experimentally the antigen is usually introduced by parenteral routes, meaning routes other than by way of the alimentary canal, such as intravenous, intraperitoneal, subcutaneous, intrathecal, intraocular, and by other similar pathways. The nature of antigens and antibodies will be discussed in the subsequent chapters, but it may be said here that both are of protein nature. Every soluble complete protein, with the exception of the racemized protein of Dakin, may serve in at least some degree as an antigen. The proteins employed are for the most part native, but synthetic proteins may also act as antigens. Wells states that "of the cleavage products of proteins it is certain that none of the amino-acids and simple polypeptids can act as antigens, and it is not yet fully established that even such large complexes as the proteoses are antigenic, although there is some evidence in favor of this view." There have been numerous reports of the use of lipoids as antigens, but this relation has not been definitely established. If lipoids are obtained from animal tissues favorable results may be obtained, but in none of these experiments is it proven that the lipoids are entirely free from proteins. Ford has successfully employed a hemolytic glucoside obtained from the poisonous mushroom *amanita phalloides* as an antigen for the production of an anti-hemolysin, but this is the only well-established exception to the general rule that antigens are of protein nature.

Actively Acquired Immunity.—This may be produced by actual infection of an individual during a period of good health by the virus of the disease to which he is to be immunized. The classical example of this form of immunization was the practice for many centuries of inoculating smallpox into the healthy, so as to induce a mild attack of the disease. The danger lies in the uncertainty of action of the virus, since apparent health does not necessarily presuppose resistance to any special disease. If the virus can be measured in some way so that an extremely small amount can be inoculated, the procedure is somewhat safer. Protection against Texas fever in cattle has been practised by permitting nursing calves to be

bitten by a small number of infected ticks or by injecting intravenously a small amount of blood from an infected animal.

Somewhat similar to the above examples is infection with attenuated virus. Such attenuation may be obtained by prolonged cultivation on artificial media, by heat, by passage through animals, by desiccation, by the use of chemical agents, and by pressure. If heat be employed for attenuation, rather than for killing the organisms, it must be properly adjusted. Toussaint employed this method in his early experiments with anthrax in which he heated infected blood to 55° C. for ten minutes. This method, however, is not reliable, probably because of variations in the resistance of individual members of a culture of any given organism. Heat may be applied also during the cultivation of organisms upon artificial media, a method practised by Pasteur in producing anthrax vaccine. The heat must be of such a degree as to permit growth of the organisms, but at the same time reduce the virulence. As has been pointed out before, the cultivation of organisms upon artificial media through many generations leads to a reduction of virulence. This latter method was employed by Pasteur in the development of the vaccine for chicken-cholera. The attenuation of smallpox virus by passage through the calf so reduces virulence that the virus may safely be inoculated into man. Pasteur found that the virus of swine erysipelas could be attenuated by passage through rabbits, and it is well known that the passage of rabies virus through dogs and through monkeys reduces its virulence. An excellent example of attenuation by desiccation is found in the preparation of anti-rabic vaccine. For this purpose the virus is raised by passage through rabbits to a standard degree of virulence, the "virus fixe." The spinal cord of a rabbit so infected is desiccated at 25° C. over KOH. This method of attenuation is so delicate that there are distinct variations in virulence between fragments dried for five, six, and seven days, as well as virus dried for thirty-five, thirty-six, and thirty-seven days or intervening periods. The longer the desiccation the greater the reduction of virulence and the greater the safety of the inoculation. Attenuation by the use of chemicals, such as phenol, potassium bichromate, and sulphuric acid, has been practised. Chemical attenuation may also be applied to toxins, as in the use of iodine terchloride and potassium iodide. A pressure of eight atmospheres at a temperature of 28° to 39° C. has been employed for the attenuation of anthrax cultures, but is probably not widely applicable, is difficult, and possesses no superior advantages.

Immunization with Dead Bacteria.—In the study of immune processes it was ~~finally~~ found that killed bacteria could be used for the production of immunity. The organisms may be killed by heat or by chemicals. In either case, it is necessary so to apply these agents as to kill the organisms without destroying their proteins. The use of heat sufficiently high to destroy spores leads to destruction also of the proteins, and therefore the method does not apply to

spore-bearing organisms. Those organisms which do not produce spores can be killed by heat of 58° to 60° C. for thirty to sixty minutes, and this degree of heat does not alter the character of the proteins. The chemicals most frequently employed for killing bacteria so as not to alter the proteins are formaldehyde and phenol.

Immunization with Bacterial Products.—In addition to the use of dead bacteria, as indicated above, it has been found possible to produce immune reactions by the use of extracts of the organisms, these extracts containing a considerable amount of bacterial protein. Immunization of this sort leads to the formation of anti-bacterial sera which agglutinate the bacteria or precipitate bacterial extracts. It is possible also that this method of immunization leads to the formation of other immune substances. How far protein, either in solution or in the bodies of bacteria, may be broken down and still be capable of leading to the formation of immune bodies is a question that has been extensively studied. Certainly any change that breaks up the protein into its fundamental amino-acids is likely to destroy its antigenic properties. Simple fractionation by means of salting still leaves sufficient native protein to serve to immunize.

Of bacterial products which have been employed for immunization none is more important than those poisonous bodies called toxins. In the classification of toxins we have referred to the true toxins or exotoxins and to the endotoxins. There is little support for the belief that endotoxins as such, except in rare instances, can produce immune substances. On the other hand, the production of a neutralizing antitoxin against the exotoxins has constituted one of the most brilliant chapters in the study of immunology, and it will be given discussion in the chapter on toxins and antitoxins. The use of toxins as antigens involves the employment of these substances in non-fatal doses, their attenuation by chemical and physical means, or their primary neutralization by means of previously prepared antitoxins. In experimental work on animals the first two methods are commonly employed and may be combined with the third method. In man immunization by the use of toxins is practised mainly in connection with active immunization to diphtheria. The combination between toxin and antitoxin is not in the nature of a fixed and final reaction, and under certain circumstances partial dissociation may occur. The active immunization of man by the use of neutralized mixtures of toxin and antitoxin appears to provide conditions whereby dissociation progresses gradually, and the toxin is liberated in such small amounts that it does no harm and yet induces in the body antitoxin formation. In the meantime the individual is protected by the antitoxin simultaneously dissociated. Recent studies make it appear that several organisms which formerly were supposed to produce only endotoxins elaborate in addition true toxins, and some of the earlier studies supporting the assumption that antitoxins could be produced by these

endotoxins are probably fallacious, because of the mixture of unrecognized exotoxins, the latter producing the immune reaction.

Active immunization may be produced not only by toxic substances elaborated by bacteria, but also by toxic substances produced in animal life, such as snake venoms, spider poisons, and similar substances. Higher plant poisons, such as ricin, abrin, croton, etc., may produce specific neutralizing antibodies. The practical value of the antitoxins prepared against bacterial toxins and against the venoms produced by animals is such as to have added greatly to the combating of poisoning by these substances.

Passive Immunization.—In active immunization the animal manufactures within its own body immune substances which serve to protect against and combat infection. Passive immunization, however, utilizes these immune substances, through the transfer of blood serum containing the products of active immunization. The most common example of passive immunization is found in the therapeutic use of diphtheria antitoxin. For practical purposes the diphtheria antitoxin is manufactured in the body of the horse. The injection of immune horse serum transfers to man the immunity actively produced in the horse. Passive immunity of this sort serves to protect against infection, and until the possibility of active immunization of man against diphtheria was demonstrated, the former method was widely employed for protection of exposed individuals against diphtheria. This method of protection has the great advantage of quickly conferring immunity and is widely employed when time does not permit the use of methods for developing active immunity. After the disease has developed the use of immune serum to combat the infection has the utmost value. In the case of tetanus antitoxin the protective value of prophylactic injections has been amply demonstrated, but in this instance the great affinity between nerve tissues and tetanus antitoxin is such that the therapeutic use of tetanus antitoxin after the disease has developed has not given such beautiful results as has been true of the serum treatment of diphtheria. Much encouragement has recently been afforded by the use of similarly prepared antitoxins against the toxin of the bacillus of gas-gangrene, and there is little doubt that the methods may be much more widely employed as it becomes possible to demonstrate the formation of true exotoxins by other bacteria. Not only may advantage be taken of substances produced by artificially acquired immunity, but in certain instances it is feasible to use the blood serum of individuals who have acquired immunity by survival of an attack of certain diseases. In this field, however, the facts have not been accumulated in sufficient number to justify unqualified approval of the method.

Passive immunity may be not only antitoxic in character, but also anti-bacterial. Anti-bacterial immune sera have been prepared against the streptococcus, the meningococcus, the pneumococcus, and other organisms. The success with passive immunization by


the use of these sera has not always been so clear cut as in the case of antitoxic sera. Nevertheless the use of anti-meningococcus sera has reduced the mortality of epidemic cerebrospinal meningitis from 75 or 80 per cent. down to 35 or 40 per cent. or lower. The results with anti-streptococcus sera have been variable. Although the early reports of the use of anti-pneumococcus sera were highly encouraging, later study has thrown some doubt upon the value of this method of treatment. Much further study of the subject is required before a definite conclusion can be reached.

Theories of the Nature of Immunity.—In the early study of immunity numerous hypotheses were advanced as to the action and development of immune bodies. It was known, for example, that when bacteria are grown for a long time upon a culture medium certain substances are produced which have a deleterious influence upon the further growth of the organisms and may actually lead to their death. It was easy to assume, therefore, that recovery from an infectious disease might be due to the development of similar antagonistic substances within the infected host. Another theory was to the effect that bacteria growing in the body utilize and exhaust the specific nutritive substances necessary for their growth and then die. It was also thought that the death of bacteria in the body was due to changes in reaction of the blood, and further that altered osmotic conditions changed the permeability of cell membranes so as to permit ready entrance of poisonous substances. These theories, however, could not withstand the demonstration of passive transfer of immunity, the production of immunity by the use of killed organisms, or perhaps more important, the clear demonstration of immune reactions *in vitro*.

The Ehrlich Side-chain Theory.—As more and more facts were added to the knowledge of the subject, Ehrlich propounded his side-chain theory. This was based upon the law of Weigert, which states that when animal cells are required to repair an injury they not infrequently exceed the absolute necessity for repair and produce tissue in excess. Ehrlich, therefore, hypothesized that the injurious substances of infection demand of the cells the formation of protective bodies, and that the cells respond to this demand in such excess that the protective bodies are formed in amounts not only sufficient to meet the requirements, but in such excess as to free circulating immune substances in the blood. This hypothesis introduced an entirely new terminology into the subject. It was supposed that cells normally possess certain specific receptors or combining groups for the injurious substances much as a structural chemical formula exhibits free valencies on the part of certain elements or groups. When all these combining groups of the cell are utilized and uncombined poisonous material exists in the circulation, the cell produces and liberates additional receptors even in excess of demand. These free receptors constitute the circulating immune substances. The study of immune substance demon-

strates somewhat variable activities. For example, it was found that antitoxins operate differently from other immune substances; that agglutinins and precipitins operate in a special fashion which is practically identical for both substances; and that cytolytins, including the lytic bodies for bacteria as well as for animal cells, require the presence of fresh serum containing the so-called complement or alexin. The specific cytolytins were found to be similar to certain other substances which are now referred to as complement-fixing bodies. Finally the discovery of opsonins and tropins showed that there is in all probability a fourth group or sub-group of these immune substances.

The Ehrlich Classification.—Ehrlich, on the basis of the general outline given above, divided the immune bodies into three groups, depending upon demonstrable differences in their nature. He found that the receptors in some instances are not immunologically simple bodies, but that even in this sense they show varying degrees of complexity. In the more complex forms the actual receptor or combining groups constitute only a part of the immune substances, and he therefore applied a more comprehensive term, the haptines. He included in the haptines of the first order the antitoxins, in the haptines of the second order the agglutinins and precipitins, and in the haptines of the third order the cytolytins and other amboceptors. The early studies of antitoxins made it appear that the neutralizing effect of these substances was similar to the neutralizing action of alkalies and acids, but it was subsequently discovered that such combinations may be, at least in part, dissociated. It was then found that toxin may undergo a variety of changes as the result of preservation. Subsequently it was learned that the antitoxin would combine not only with the toxin, but with its degeneration products. This has complicated the conception considerably, and we may say in brief that according to the Ehrlich conception the antitoxin constitutes a simple receptor or combining group capable of entering into combination with a special combining group in the toxin called the haptophore. In order to account for the combination of antitoxin with the degeneration products of toxins it was necessary to assume that *the toxin* exhibits its essential combining property in the haptophore group and that the toxin also possesses a toxophore group, serving to give it its poisonous character and partly destroyed during preservation or by certain degrees of heat. The second category of Ehrlich includes the agglutinins and precipitins. In the study of these substances it was found that the agglutinins and precipitins may be deprived of the agglutinating and precipitating properties by preservation or by the application of certain degrees of heat. Ehrlich, therefore, conceived the idea that in this instance we have to deal with a somewhat more complicated haptine containing a combining group and a so-called zymophore group, the latter leading to the special reaction. These constitute the receptors or haptines of the second order. This assumption is neces-



sary, because even although the agglutinating and precipitating properties are destroyed by heat or other means, nevertheless, there remains a group capable of entering into combination with the antigenic substances, so that the addition of a complete agglutinin or precipitin produces no effect. The third category of Ehrlich includes the receptor or haptine which has been named by Ehrlich the amboceptor, and by Bordet the sensitizer. In this instance the receptor produced by the cell is conceived as a body possessing two combining groups, one serving to combine with the antigen and the other serving to combine with complement. These two groups have been called the cytophilic group and the complementophilic group. The complement is a thermolabile substance which has little or no capacity for combining with the antigen. Accepting Ehrlich's hypothesis, this haptine of the third order constitutes an intermediary body through the action of which the complement is brought into contact with the cells, be they bacterial or animal, so as to lead to solution. Bordet, however, believes that the immune body enters directly into combination with the antigen, thereby "sensitizing" it so that the complex is operated upon by the complement, or as he calls it, the alexin. The discovery of the phenomenon of complement-fixation demonstrated that a similar substance may operate in the presence of dissolved protein and complement, so as to engage the complement in such a fashion that it is not available for other reactions. In these reactions the participation of the complement is an essential and necessary condition of the reaction. The original Ehrlich theory could not consider the subsequently discovered opsonin or tropin. This substance prepares bacteria and other cells for phagocytosis. It was at first supposed to be a simple immune substance, but as the study of its activity progressed it was found that the presence of complement increases its activity, although this latter body is not essential and necessary. We, therefore, propose to consider the opsonin as belonging essentially to the haptines of the third order. The way in which this differs from the original haptine of the third order is simply in the fact that complement may or may not be utilized in the reaction. In order to differentiate we suggest that the amboceptors of Ehrlich be looked upon as "obligate" amboceptors and the opsonin be regarded as a "facultative" amboceptor.

Recent Criticism of the Ehrlich Hypothesis.—Following the earlier discoveries of immune phenomena numerous studies were made of the chemistry of immune substances and immune reactions. These will be discussed in the chapters on the special immune reactions. It may be said at this time that many objections have been raised to the Ehrlich hypothesis, particularly as the study of physical chemistry, more particularly that part which refers to colloids, has advanced. As will be seen from the brief review of the Ehrlich hypothesis given above, this investigator was much influenced by the status of chemistry which prevailed when he announced his

views. The idea predominated that the immune reactions resemble the more or less fixed changes which are seen in the chemical reactions of crystalloids. As it was found that practically all immune substances are colloidal in nature and either are proteins or are very closely related to proteins the similarity of the immune reactions to colloidal reactions became more and more strongly emphasized. In fact, in a general way, practically all immune reactions parallel in their general phases similar demonstrable reactions with colloids. There is but one feature of immune reactions which has not yet been explained on the basis of colloid chemistry, namely, their specificity. This does not mean, however, that further investigation will not clear up this phase of the problem. It is but fair to say that the Ehrlich hypothesis provides an excellent basis for the classification of immune phenomena, but as will be shown subsequently, the conception underlying the Ehrlich hypothesis is not adapted to the more modern views of the mechanism of immune reactions. The combination of toxin and antitoxin shows numerous features not to be explained by the simpler reactions of crystalloids. The same is true of agglutination, precipitation, cytolysis, complement-fixation, and anaphylaxis.

Specificity of Immune Reactions.—The antitoxin elaborated in the response to injections of diphtheria toxin or to the presence of the disease itself is a substance which reacts only with diphtheria toxin. The agglutinins and precipitins produced by injection of bacteria and of dissolved proteins act most powerfully upon the substances used for injection. In this case, however, these immune bodies may also react less strongly with other closely related bacteria or proteins. Cytolysins induced by the injection of certain cells react strongly with those cells, but also less strongly with closely related cells. This phenomenon of reaction with closely related bodies is spoken of as the group phenomenon and may be exhibited also in connection with complement fixation and anaphylaxis. Even where purified proteins are employed the same phenomenon may be observed. In spite of the group reaction, however, the immune substances are most highly specific for their special antigens. Specificity has been employed for the detection of particular proteins of animal species, of bacterial species, and it has lent support to the Darwinian theory of species relationship and evolution. Much thought and study has been given to the resemblance between immune substances and enzymes, but in no sense can enzymes be said to have the same specific character as immune bodies. There is no satisfactory explanation of specificity. Why the injection of red blood-corpuscles of the sheep should induce the formation of a hemolysin capable of dissolving the red cells of the sheep but not of other animals, except in minor degree of those closely related to the sheep, cannot be explained. As can readily be understood, specificity involves the use of special antigens and the formation of more or less specific immune substances. The wide

range of possibility in this connection is indicated in the building-stone theory of Abderhalden. Bearing in mind that practically all immune substances are protein in nature and that proteins are made up of numerous amino-acids, Abderhalden calculated that twenty amino-acids could be so combined as to form 2,432,902,008,176,640,000 different compounds. He illustrates this possibility by stating that if three amino-acids are building stones which may be designated A, B, and C, they can be grouped together so as to form six different combinations, ABC, ACB, BCA, BAC, CAB, and CBA, and that four building stones can form twenty-six such combinations and so on until the enormous possibility of different combinations of twenty amino-acids is reached, as illustrated in the figures given above. Chemically no such enormous number of proteins is known, but if immune specificity could be shown to depend upon slight differences of molecular arrangement, Abderhalden's figures indicate the number of immunologically specific proteins obtainable. Taking for granted the phenomenon of specificity, that of the group reactions can be more readily explained. In this case it is assumed that in the proteins of closely related species there is some group of molecules common to these species, and further that the formation of immune substances in response to injections of this common group leads to the production of a substance which may react with the common group. In each species, however, there is in addition to the common group special groups which determine the specificity of the substance as an antigen as well as the production of an immune substance with a higher degree of affinity for the combined groups of the particular species than for the common group.

Non-specific Therapy of Infectious Disease.—As a result of the extensive studies of infectious disease various modes of treatment have been elaborated. It is well understood that the organism offers resistance to these infections and that the support of circulation and excretion by simpler pharmacological methods aids materially in the treatment. Not only is this true, but the investigation of various drugs has determined the specific chemo-therapeutic treatment of infections. Examples of this are seen in the use of quinine in malaria, arsenic in trypanosomiasis and spirochetosis, and of emetin in amebiasis. The treatment based more particularly upon immunological methods has been largely specific, but more recent studies have given encouragement in the use of certain non-specific methods of treatment. It was found, for example, that the use of typhoid vaccine is of value not only in the treatment of typhoid fever, but in other diseases, and typhoid vaccines either in the form of the usual killed organisms or organisms sensitized with specific immune sera have produced beneficial results in such diseases as acute articular rheumatism, sub-acute and chronic arthritis, and in certain other infections. Similarly the use of blood serum, of pure proteins, of leucocyte extracts, of fibrin derivatives, and of certain other protein derivatives has appeared to be beneficial. It is not to

be assumed that this method of non-specific treatment is of conclusively proven value, but the effects observed in a certain percentage of cases offers the hope that the method may be so perfected as to give improved results.

The general reaction following subcutaneous injection of these substances may or may not be severe, but if they are administered by the intravenous route the reaction is likely to be pronounced. Frequently a chill appears and almost all cases develop fever which may be very high. Sometimes there is a general feeling of discomfort associated with headache and nausea. In typhoid fever it is reported that hemorrhages not infrequently occur as the result of the therapeutic use of sensitized and of non-sensitized typhoid vaccine. This does not appear in other diseases, and although protein substances and their cleavage products, upon injection, tend to decrease the coagulation time, yet the use of blood serum in the treatment of hemophilia often has a favorable effect in preventing hemorrhage. In addition to the possibility of hemorrhage in typhoid fever there are definite contraindications to this form of therapy in pregnancy, in patients with organic heart disease, and in those with high blood-pressure. The influence of this non-specific method of treatment is not clearly understood. The question as to whether or not the known forms of antibodies are liberated or stimulated has been studied by numerous workers with contradictory results. Some have found an increase of agglutinins and precipitins for the specific organisms concerned in the disease, following non-specific protein injections, but this is contradicted by other workers. Regardless of the question of stimulation of special immune bodies it is important to know what other protective influences may be set at work.

Fever is a common incident of the injection of proteins or protein products, especially when they are given intravenously. This is sometimes accompanied by leucocytosis, but neither leucocytosis, marked acceleration of pulse-rate, nor the other clinical accompaniments of fever necessarily appear. It has been demonstrated that increased temperature aids in the production of agglutinins and bacteriolytic substances. In most instances the degree of temperature reached in fever has no deleterious effect directly upon the bacteria concerned, except possibly in the case of infections with the gonococcus and with the spirochete of relapsing fever. It has been suggested that high body temperature may favor the combination of the antigen and immune substances, but this has not been conclusively demonstrated. The injection of proteins may lead to an increase in the number of circulating leucocytes, although this is not invariably the case. The influence of such a hyper-leucocytosis in combating infection is at least partly because of the fact that these cells ingest and destroy bacteria. Nevertheless, certain infectious diseases, such as typhoid fever, may run their course without exhibiting leucocytosis, and it is therefore not essential for recovery that the leucocytes be increased in number. It must be

pointed out, however, that phagocytosis is not the only way in which an increase of leucocytes may operate beneficially. The studies of Hiss and Zinsser indicate that extracts of leucocytes have a beneficial effect on infections and others have confirmed these results. Bail claims that a fresh emulsion of leucocytes will aid in neutralization by the specific anti-serum of endotoxin obtained from cholera vibrios. Jobling and Bull, however, demonstrated that leucoprotease "will destroy the toxic extracts of typhoid bacilli and meningococci, and it is not improbable that a similar explanation will apply to the results obtained by Bail."

There are other possible changes in the blood as the result of the injection of protein. The work of Jobling and his collaborators has thrown great light on the alterations of ferments and anti-ferments in the blood under a wide variety of conditions. The injection of various substances is almost invariably followed by a considerable mobilization of the serum ferments, more particularly the protease, and usually also the esterase. The value of the protease is probably in the direction of breaking down toxic split-protein products, which probably originate during the course of infectious disease, as the result of the splitting of bacteria and perhaps also of the body proteins. Protease does not act directly upon living bacteria, but it is to be considered possible that the esterase may break up the lipoid or lipoid-protein surface of the bacteria and therefore aid in their destruction. If we concede that the toxic protein split products aid in the virulence of bacteria it is possible that even although the protease simply breaks down these products into simpler non-toxic substances and does not directly attack the bacteria, yet the relief to the body afforded by this detoxifying action may assist it more permanently in combating disease. In certain states, such as pregnancy, in disease such as cancer, and in the course of vaccine treatment the anti-ferment titer of the blood has been found to be high. Jobling and Peterson found that the anti-ferment power of the blood depends upon the amount of unsaturated lipoids present in highly dispersed phase in the serum and Bogolemez suggests that lipoids may serve to inhibit toxins, as is true in relation to the toxin of bacillus botulinus. Anti-ferment is not increased following protein injections and plays no part in the non-specific therapy of infectious disease, but inasmuch as the change may be seen in immune states, such as that following vaccination, it may be of importance in non-specific resistance to infection. In addition to the changes in ferments Jobling has found that the injection of non-specific proteins may produce changes in the viscosity of the serum. It is known that if precipitates are formed in serum by the action of a specific precipitating serum, conditions favorable to protease activity are produced and the changes in viscosity produced by protein injections may similarly aid proteolytic activity. These changes in ferment content and physical character of the serum are of short duration and are probably contemporaneous with the chill and fever.

They do not directly account for permanent improvement seen in many patients, but if they rid the body of toxic substances for a short period of time the natural resistance may thereby become more effective than would otherwise be the case.

The Site of Antibody Formation.—Aside from a few fairly well-established facts the problem as to exactly where antibodies are formed still remains obscure. In general it is assumed that antibodies are not products of simple inversions of the foreign protein substances parentally introduced or as particular functions of special organs, but are the result of general cell reactions on the part of the host. Much evidence points to the lymphatic organs, the spleen, the liver, and the bone marrow as places where antibody formation is most active. Metchnikoff thought that antitoxins and bacteriolysins originate in the lymphatic organs and more particularly in the spleen and the bone marrow. Bordet attempted to show that bacteriolysins are derived from the leucocytes. Pfeiffer and Mark injected dead cholera spirilla into animals, exsanguinated these five days after the injection, and found the antibodies more concentrated in the spleen than in the blood serum itself. These authors also found that after a single injection of these organisms, the spleen, the bone marrow, and the lymph-nodes contained the specific antibodies before they could be detected in the blood, and further that as time passed these tissues became less active in spite of the fact that the bacteriolysins increased in the blood. Deutsch corroborated these findings with bacillus typhosus and Castellani with bacillus dysenteriae. These authors agree, however, that the spleen is not essential, since its removal but slightly inhibits the formation of antibodies. Hektoen's experiments demonstrated that in dogs splenectomy just before and after the injection of alien blood-corpuscles was followed by a much lower, but otherwise typical antibody curve, than is usually the case in dogs under normal conditions. London also reported a decreased formation of hemolysins after splenectomy, but this work has been contradicted by Yakuschewitch. Karsner, Amiral, and Bock found that splenectomy produces no change in hemopsonins of the circulating blood that is clearly demonstrable by *in vitro* test, and that the blood from the spleen is no richer in hemopsonins than is blood from other organs. Carrel and Ingebrigsten have produced hemolysins in the growing embryonic spleen. More recently Przygode succeeded in producing precipitins *in vitro* by culture of splenic tissue, and Müller by transplanting splenic tissue from guinea-pigs, previously injected with sheep corpuscles, into the peritoneal cavity of normal guinea-pigs. It seems to us that since the spleen is an organ physiologically designated for the destruction of erythrocytes and also of other foreign substances through the activity of its hemophages, splenic tissue on transplantation will carry with it much antigenic substance. Whether or not these hemophages participate in antibody production is at present difficult to say.

For rapid production of antibodies Viollé injected organisms directly into the gall-bladder. This fact is of interest because it indicates a possible function of the liver in the production of immune bodies. Müller claims to have been able to stimulate the formation of hemolysin in liver tissue suspended in Ringer's solution outside the animal body. By perfusing the organ with solutions containing iodine (iodipin) the effect was augmented, and he believes that in the normal animal the iodine of the thyroid may play a certain rôle in stimulating this special activity of the liver. Gay and Rusk found no evidence to uphold the supposed influence of iodipin. Hektoen and Carlson believe that both the spleen and liver are equally concerned in antibody formation, but Hektoen and Curtis found that in rats removal of about one-half of the liver appears to have no effect on the development of hemolysin for sheep corpuscles. The liver, just as the spleen, possesses highly active phagocytic endothelial cells which may play an important rôle in the production of antibodies.

Numerous authors have shown that agglutinins appear in the blood stream before they are present in the extracts of any organ. The question, however, of whether or not the leucocytes are involved in this generative process is a matter of considerable controversy. Achard and Bensaud and others controvert the leucocytic or local origin of agglutinins, whereas Cantacuzène and also Swerew support this local origin in the formation of precipitins; they noted a hypoleucocytosis followed by a marked hyperleucocytosis, which they think is responsible for the liberation of precipitins. Petit and Carlson, Vaughan, Cumming, and McGlumphy found that substances like egg-white and serum disappear quickly from the circulating blood; in fact, within a few hours after the introduction of these substances. Gay, however, has shown by means of complement-fixation that even in immune animals such antigens are demonstrable after twenty-four hours, but not after forty-eight hours. It was not possible to demonstrate the antigen by the fixation method in organs like spleen, lymph-nodes, liver, kidney, and muscles, either at the time antigen was present in the blood or twenty-four hours thereafter. That the cells lining the blood-vessels may have certain powers of antibody production may be shown by the fact that a blood-vessel from an animal which has received several injections of sheep erythrocytes and which has been dissected out soon after death of the animal and washed free from blood, has the power to hemolyze a suspension of fresh, non-sensitized sheep cells (Van Calcar). Kraus and Levaditi furthermore have shown that there exists a certain relationship between precipitins and the number of circulating leucocytes. Acute loss of blood profoundly affects antibody production. The earliest observations seem to have been those of Roux and Vaillard. They found that in horses actively immunized against tetanus toxin, bleeding causes a drop in the anti-toxin content in the blood, followed by a sharp rise in a short time.

By continuous daily bleedings Hahn and Langer recently succeeded in increasing the agglutinin content 250,000 times its original value. Similarly Madsen and Tallquist have shown that certain poisons which destroy erythrocytes may increase the production of antibodies possibly by the action of the same mechanism as that whereby hemorrhage stimulates antibody formation. Rusk has found that animals intoxicated with benzol produce hemolysins and precipitins much less efficiently than normal animals. Since benzol affects particularly the bone marrow and the lymphatic apparatus, this evidence points in favor of the view that these tissues are largely involved in the production of hemolysins and of precipitins.

According to Hektoen and Curtis, adrenalectomy in normal dogs and in dogs at the height of the antibody curve after the injection of rat corpuscles does not cause a decrease in the antibody content of the blood serum. Gates was able to remove approximately three-quarters to seven-eighths of the adrenal tissue of guinea-pigs without causing symptoms of adrenal insufficiency. Guinea-pigs thus treated were injected with bacillus typhosus and with hen corpuscles, and the results demonstrated that adrenalectomy had no influence upon the rise or persistence of antibodies in the blood, and therefore the adrenals appear to play no essential part in the mechanism of antibody production.

The results of Tjeldstad had shown that thyroidectomy failed to influence antibody production. Similar observations were recorded by Hektoen and Curtis, and others, but Frouin was more conservative in his conclusions, and Garibaldi has recently renewed an interest in this matter by reporting that the hemolytic titer of the serum of thyroidectomized rabbits is much higher than that of his control animals, therefore concluding that thyroidectomy definitely favors antibody production.

We know from the experiments of Wassermann and Takaki that brain substance neutralizes tetanus toxin, but this fact does not indicate this organ to be of much importance in the production of antibodies. In fact, we have learned from the experiments of Loewi and Meyer that injection of toxin into the nervous system produces an increased susceptibility of the animal rather than increase of resistance.

Production of Antibodies at Site of Injection.—Certain experiments indicate that antibodies may also be produced at the place of introduction of the antigen. Römer and also von Dungern have shown that immunization by way of the conjunctiva or anterior chamber of the eye results in the formation of antibodies in the aqueous humor before they can be demonstrated in the blood. These experiments also demonstrated that the opposite eye produces no antibody. Wassermann and Citron ligated a rabbit's ear at its base after a subcutaneous injection of bacteria. The ligation was left for several hours, and after nine days the bactericidal titer of the blood serum determined and the ear amputated. An immediate and rapid drop of antibody in the blood which occurred after the ampu-

tation indicates that the main source of antibody formation was removed or the absorption of the antigenic substances entirely stopped. Forsmann and Lundström studied the curve of production of botulinus antitoxin following single intravenous or subcutaneous injection of the toxin. The curve following the subcutaneous injection reached its highest level on the fifteenth day, while that following the intravenous injection attained the maximum on the tenth day. It must be inferred that the subcutaneous method of injection introduces the factor of slow absorption, but it is also possible that some local factor may enter into the phenomenon. Immunization of horses with diphtheria toxin results in a greater yield of antitoxin when the horses are injected subcutaneously, but this does not necessarily prove a local production of antitoxin at the site of inoculation. Local cellular participation in immune reactions will be discussed further in the chapter on hypersusceptibility. There is little doubt that local reactions are of significance and that absorption may be influenced by local changes. The production of circulating antibodies in any considerable amounts undoubtedly requires more extensive cellular activity than that about the site of local inoculations.

CHAPTER IV

TOXINS AND ANTITOXINS

GENERAL NATURE OF TOXINS.

THE BACTERIAL TOXINS.

CLASSIFICATION.

PATHOLOGICAL EFFECTS.

FORMATION OF ANTITOXINS.

TECHNIC OF PRODUCING DIPHTHERIA TOXIN.

THE NATURE OF ANTITOXINS.

STANDARDIZATION OF DIPHTHERIA ANTITOXIN.

THE MINIMUM LETHAL DOSE (M.L.D.).

THE L₀ DOSE (LIMES NULL).

THE L₊ DOSE (LIMES DEATH).

TITRATION OF DIPHTHERIA ANTITOXIN.

THE TOXIN ANTITOXIN UNION.

THEORIES OF UNION.

EHRLICH THEORY.

LAW OF MASS ACTION.

THE DANYSZ EFFECT.

THERAPEUTIC USE OF DIPHTHERIA ANTITOXIN.

VALUE.

MODES OF ADMINISTRATION.

NATURAL IMMUNITY TO DIPHTHERIA.

THE SCHICK TEST.

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TETANUS TOXIN AND ANTITOXIN.

TETANOTOXIN.

TETANOSPASMIN.

ROUTE OF ABSORPTION OF TOXIN.

THERAPEUTIC USE OF TETANUS ANTITOXIN.

PROPHYLAXIS.

TREATMENT OF THE DISEASE.

DYSENTERY TOXIN.

THERAPEUTIC USE OF DYSENTERY ANTISERA.

BOTULINUS TOXIN.

THE USE OF IMMUNE SERA IN BOTULISM.

GAS BACILLUS TOXINS.

THE USE OF IMMUNE SERA IN GAS GANGRENE.

TREATMENT OF THE DISEASE.

PROPHYLAXIS.

BACTERIAL HEMOTOXINS.

STAPHYLOLYSIN AND ANTILYSIN.

THE PHYTOTOXINS.

RICIN.

ABRIN.

CROTIN.

CURCIN.

PHASIN.

THE ZOÖTOXINS.

SNAKE VENOMS.

SCORPION AND SPIDER POISON.

CENTPEDE POISON.

BEEES, WASPS, AND HORNETS.

TOADS, FROGS, AND SALAMANDERS.

POISONOUS FISH.

EEL SERUM.

PARASITIC PROTOZOA.

MAMMALIA.

ANIMAL SERA.

General Nature of Toxins.—Toxins are soluble poisonous products of life processes, which on injection into animals lead to the formation of antitoxins. A corollary of this definition sometimes insisted upon is that the injurious effect of these toxic bodies must be preceded by an incubation period, but in certain instances this incubation time is a matter of minutes or hours, as is the case with certain snake poisons. The toxins are divided according to their origin into phytotoxins, produced by vegetable life, and zoötoxins, produced by animal life. The most important of the phytotoxins are the bacterial toxins, but the group includes also ricin, abrin, croton, robin, and curcin. Certain of the higher plant poisons which produce the varieties of "hay fever" in susceptible individuals were formerly considered as toxins, but this view has now been discarded. The poison of *rhus toxicodendron* (poison ivy) and of *rhus diversiloba* (poison oak) might be considered a phytotoxin, but is chemically a glucoside and does not produce antitoxin. The poisoning of non-edible mushrooms is due, in the case of *amanita muscaris* and *helvella esculenta*, to definite chemical compounds, muscarine and helvellic acid, which do not produce antibodies. In the case of *amanita phalloides* there are two substances of toxic nature, a thermolabile hemolytic glucoside capable of producing an anti-hemolysin, and a thermostabile toxin of unknown composition and incapable of producing a definite immune body. The most important of the zoötoxins are the snake venoms, but this group also includes the poisons of spiders, scorpions, centipedes, bees, wasps, hornets, dermal glands of toads and salamanders, various sera, notably that of the eel, and certain poisonous fish.

Nicolle, Césari, and Jouan divide the toxins into those that appear in the form of definite secretions, as snake venoms, those which are determined by logical inference, as the toxins in microbial filtrates, and those which are obtained by simple maceration, expression, grinding, or autolysis, the endotoxins. Experiments with the endotoxins are performed in large part with the microbial bodies, and therefore these workers refer to the endotoxins as solid toxins.

The bacterial toxins are synthetic products of the life of the organisms themselves. It was thought for years that the bacteria could synthesize the protein toxin from simple nitrogen-containing compounds. More modern studies oppose this view and state that more complex substances, such as proteoses and polypeptids, are essential. It seems certain that nothing less complex than the amino-acids can be synthesized, and recent studies indicate that diphtheria toxin is not a synthetic product, but rather a catabolic substance elaborated by the bacteria only in the presence of amino-acids and certain additional substances, probably of the nature of vitamins. They are thus to be distinguished from the ptomaines, which although products of bacterial growth, are in reality formed from the culture medium and vary according to the medium rather than according to the organism. The chemical nature of the bacterial

toxins is uncertain, but they appear to be more closely related to the proteins than to any other known substance. They diffuse through membranes less slowly than do proteins, and therefore are presumed to have a smaller molecular size. On the other hand, they are digested less readily than proteins. Like proteins they are electro-positive colloids, and are precipitated by protein precipitating agents, such as ammonium sulphate. As against this is the statement that toxins may be so far purified that they do not give the protein reactions. They resemble enzymes in that both are colloids, both thermolabile, dialyze with difficulty, lose strength in passing through porcelain filters, resist drying and dry heat, resist low temperatures, both produce antibodies, both deteriorate after standing in solution with loss of zymophore group, but without loss of haptophore or combining groups. The difficulty of establishing the toxins as enzymes lies in the fact that neither toxin nor enzymes have been isolated in the pure state. Furthermore, they do not act according to the same chemical laws, the enzyme operating repeatedly to produce a large effect in the course of time and the toxin acting in almost direct proportion to its quantity. In summary we may quote Oppenheimer as saying of toxins that, "we must be contented to assume that they are large molecular complexes, probably related to the proteins, corresponding to them in certain properties, but standing even nearer to the equally mysterious enzymes with whose properties they show the most extended analogies, both in their reactions and in their activities."

Toxins may be injured in a variety of ways. They may be destroyed, with certain exceptions, by moist heat at about 80° C., and resist dry heat to over 100° C. Light operates in a general way according to its intensity and penetrating power and the action is intensified by the presence of oxygen. Diffuse daylight operates slowly, but direct sunlight, X-ray, and ultra-violet rays more rapidly. They are destroyed by fluorescent substances. Oxygen and oxidizing substances injure and destroy toxins both *in vivo* and *in vitro*. Certain chemicals are injurious, as the salts of bivalent and trivalent metals, but not of monovalent metals. Certain toxins, particularly dysentery and diphtheria, may be rendered non-toxic by acids and restored to toxicity by alkali. They may be bound by fats and lipoids, as illustrated in part, at least, by the neutralization of tetanus toxin by brain substances. Enzymes, such as pepsin and pancreatic juice, as well as bile, destroy certain toxins, so that they produce no symptoms following ingestion, the striking exception being botulinus toxin. The action of digestive ferments upon toxins has recently been studied in detail by Loewi. He finds that diphtheria toxin is destroyed by pepsin and ptyalin, that tetanus toxin is destroyed by trypsin and ptyalin, but not by pepsin; and that dysentery toxin is destroyed by the action of the duodenal mucosa of rabbits, but resists digestion with trypsin, ptyalin, pepsin, and papayotin.

Classification of Bacterial Toxins.—The bacterial toxins are classified as exotoxins and endotoxins, the former appearing in the culture medium as soluble substances and the latter appearing within the bacterial bodies. These intracellular toxins can be liberated by digestion, autolysis, freezing and fine grinding, and by expression with a Buchner press. They cause the symptoms of their special diseases, and in the natural course of the disease are probably liberated either by autolysis or by the action of the enzymes of the cells or fluids of the host. The tendency to-day, however, is to accept the view that the so-called endotoxins are not produced as such, but are produced from the bacteria during the process of hydrolytic cleavage of the bacterial proteins by ferments provided by the host. Certain bacteria, such as diphtheria and tetanus, produce only exotoxins, whereas the typhoid group and certain other organisms were supposed formerly to produce only endotoxins. However, Bull has shown that certain strains of the gas bacillus of Welch produce an active exotoxin, and Ecker has shown that certain strains of bacillus paratyphosus B produce exotoxins, and this has been confirmed by Aronowitch. Kraus has shown a similar relationship in bacillus dysenteriae. Studies of Admont Clark and Felton indicate that the streptococcus hemolyticus produces a filterable toxic product answering all the requirements of a true toxin. The production of exotoxins is important for practical purposes because the endotoxins do not lead to antitoxin formation with the same degree of facility as do exotoxins. Recent studies by Olitsky and Kligler have shown that the dysentery bacillus (Shiga) produces a thermolabile exotoxin and a thermostable endotoxin, the latter not being neutralized by anti-exotoxic serum. A potent antiserum for both toxins can be developed in the horse. The exotoxin appears to have special affinity for the nervous system of the rabbit and the endotoxin operates particularly upon the intestine.

Organisms which produce exotoxins show a considerable variation of this property, but, on the whole, such toxins are more virulent and more highly antigenic than the exotoxins of those organisms which are essentially endotoxin producers. Being more highly antigenic the antitoxins produced by exotoxins are the more powerful, as is well known in the case of diphtheria and tetanus antitoxins. Nicolle, Césari, and Jouan maintain, on the basis of certain work with the bacillus of Nocard, that exotoxins and endotoxins are identical in the case of a given organism, but the more recent studies of Olitsky and Kligler quoted above would indicate that this is not true of dysentery bacillus (Shiga), and therefore not a general law.

The exotoxins include diphtheria toxin, tetanus toxin, botulinus toxin, dysentery toxin, paratyphoid toxin, and bacillus aerogenes capsulatus (perfringens) toxin. Toxins are produced also by bacillus edematiens (Weinberg), vibron septique, bacillus of symptomatic anthrax, bacillus pyocyaneus, streptococcus, and bacillus influenzae. In addition it is claimed by Kolmer and his co-workers

that they have demonstrated in pneumonic exudates a pneumococcus toxin. A number of other organisms produce lytic bodies for red blood-cells or hemotoxins, such as staphylolysin and megathერიolysin, capable of inducing the formation of antilysins. A lytic body for leucocytes is also produced by staphylococcus aureus.

The pathological effects of toxins are fundamentally seen in the production of cloudy swelling and even fatty degeneration of the parenchymatous viscera, heart, vascular muscle, liver, kidney, and secreting glands. Local inflammation at the site of injection, sometimes leading to necrosis, is a frequent finding. Diphtheria toxin may show, in cases of paralysis, myelin sheath degeneration or inflammation of nerves, and in guinea-pigs usually shows marked congestion or hemorrhage in the adrenals. Botulinus toxin leads to meningeal and even cerebral thrombosis and small hemorrhages. Both botulinus toxin and tetanus toxin have a marked affinity for the nervous system, but the effects are seen in the form of functional disturbance rather than morphologically demonstrable change, except for the vascular changes produced by botulinus toxin.

Formation of Antitoxins.—Antitoxins are produced by the repeated parenteral injection of the toxin. Parenteral injection signifies introduction into the body by routes other than absorption through the alimentary canal. The selection of the species of animal to be used depends in part on its demonstrated ability to produce antitoxin and in part in commercial establishments on the possibility of obtaining large volumes of immune serum. It is often found desirable to select a species which has natural immunity to certain toxins and by inoculation raise that immunity to a higher degree. This may be accomplished with less difficulty than if a susceptible species were used. This is true of the use of the horse in producing gas bacillus antitoxin. The same principle is employed by Kyes in using fowl for the production of an anti-pneumococcus serum, although in this case it is not clear that the serum is an antitoxic serum. Kyes states that the antiserum is antibacterial, *i.e.*, agglutinating and bacteriolytic. Especially in the case of susceptible animals and also in relatively immune animals it may be necessary either to dilute the toxin to a very high degree or to attenuate it by other means, and thus consume considerable time in developing a high degree of immunity. For immunizing guinea-pigs against diphtheria toxin Behring and Kitasato used iodine terchloride, and Roux and Martin, Lugol's solution. Fränkel heated the toxin to 60 degrees. Behring in the case of tetanus toxin used a neutralized mixture of toxin and antitoxin, gradually reducing the amount of antitoxin, and finally using unmodified toxin. In a sense this latter method has been employed by Behring and by Park for producing active immunity to diphtheria in children, although here it has been found unnecessary to use pure toxin without antitoxin to attain the desired result. It is believed that after injection there is a dissociation of the mixture, which permits the

toxin to induce active antitoxin production by the patient's own body. In the work with animals the injections are given at intervals of a few days, sometimes interspersed with rest periods of about a week, until testing of small amounts of serum shows that a satisfactory result has been attained.

Technic of Producing Diphtheria Toxin, and Antitoxin.—Some details of the technic of producing diphtheria antitoxin may well serve as an example of the general phases of the method. It was early noted that different strains of the diphtheria bacillus produced toxins of variable strength and also that the same strain showed slight variation. Finally the strain isolated by Park and Williams, now well known as Park No. 8, a strong toxin producer, was selected as a standard and is so used throughout the world. In order to obtain the best aerobic conditions, a wide-bottom flask is employed so as to expose a large surface of the medium, "bob" veal broth being selected as most desirable. The culture is planted superficially and allowed to grow for seven or eight days. It is wise to use a culture that has been grown for several generations on the surface of broth tubes, the tubes so inclined as to expose a large broth surface. This accustoms the organisms to freely aerobic conditions. After the period of growth in the flask the organisms are killed by formaldehyde or by phenol, or even by heat, and the broth filtered either through paper or through a porcelain filter, preserved with toluol and permitted to "ripen." This ripening is made desirable because of progressive deterioration of the toxin, a phenomenon which will be more profitably taken up in the discussion of the toxin-antitoxin combination.

The following table shows a scheme as actually practised for producing diphtheria antitoxin. All the injections are subcutaneous.

INJECTION SCHEME FOR PRODUCTION OF DIPHTHERIA ANTITOXIN	
July 22	6,000 units antitoxin
25	400 minimum lethal doses of toxin (see page 45)
27	800 minimum lethal doses of toxin
29	1,200 minimum lethal doses of toxin
Aug. 1	1,600 minimum lethal doses of toxin
3	2,000 minimum lethal doses of toxin
5	2,500 minimum lethal doses of toxin
8	3,000 minimum lethal doses of toxin
10	3,600 minimum lethal doses of toxin
12	4,400 minimum lethal doses of toxin
15	5,200 minimum lethal doses of toxin
17	6,200 minimum lethal doses of toxin
19	7,200 minimum lethal doses of toxin
22	8,500 minimum lethal doses of toxin
24	10,000 minimum lethal doses of toxin
26	13,000 minimum lethal doses of toxin
29	16,000 minimum lethal doses of toxin
31	20,000 minimum lethal doses of toxin
Sept. 2	24,000
5	28,000
7	32,000
9	36,000
12	40,000

Lengthen intervals by 24 hours if made necessary by severe reaction.

Trial bleeding separation of and testing of serum, September 21. After that if further immunization is necessary the dose is raised 5000 M.L.D. each injection.

The Nature of Antitoxins.—The serum thus obtained contains the antitoxin. The exact nature of the antitoxin is unknown, but chemical examination and other studies have thrown a certain amount of light upon its properties. If we can accept the division of the serum protein into fibrinogen, euglobulin and pseudo-globulin by precipitation with magnesium sulphate or ammonium sulphate, the antitoxin is found in that water-soluble fraction known as

pseudo-globulin, which constitutes about 78 per cent. of the serum protein. This fact is taken advantage of in the so-called concentration of antitoxin, in which the pseudo-globulin is thrown down by the addition of ammonium sulphate. Heinemann states that pseudo-globulins may be broken into fractions, one of which contains the antitoxin in highly concentrated form, thus making the bulk even smaller than by the use of pseudo-globulin. The precipitate is collected, dialyzed free of salt, and taken up in water, the final volume being considerably less than the original amount of serum, therefore containing a greater number of antitoxic units per c.c. than the whole serum. This does not mean that the antitoxin is necessarily a globulin, for it resists trypsin digestion in greater degree than does globulin. It is, however, an electro-positive colloid. Antitoxin is not thrown down in indifferent precipitates, and in this respect differs from the enzymes, nor does it operate in the same quantitative relations as enzymes. The large size of the antitoxin molecule is indicated by the famous Martin and Cherry experiment, which showed that if toxin and antitoxin are mixed and passed through gelatin filters the toxin appears first. The same point was brought out by Arrhenius and Madsen, who showed that toxin diffuses ten times more rapidly than antitoxin. Antitoxin is injured by moist heat of 60° to 70° C., destroyed by moist heat of 100° C., and by dry heat of 140° C.

The influence of temperature on antitoxin is of the utmost practical importance in regard to its preservation for therapeutics. Anderson has estimated the yearly deterioration at different temperatures as follows:

Temperature	Yearly deterioration
26° – 35° C.	20 per cent.
15° C.	10 per cent.
5° C.	6 per cent.



FIG. 1.—Apparatus for filtration through porcelain of small quantities of material. Between the rubber tube and the suction apparatus must be inserted a small trap to prevent entrance into the flask of water when suction is released. The trap is a salt neck bottle with an inlet and outlet tube through a rubber stopper.

McConkey has given the following rates of deterioration :

Temperature	Deterioration in 6 months
36° C.	37 per cent.
6°-16° C.	14 per cent.
Ice chest	7 per cent.

The second figures in McConkey's table indicate room temperature in winter and summer. Although the two series of investigations differ in actual figures, they serve to show that the only temperatures for satisfactory preservation are those of the ice chest. Antitoxin is destroyed by putrefaction of the serum, by acids and alkalies, by ultra-violet rays and deteriorates in solution, by exposure to light and air. Ingestion into the alimentary tract destroys antitoxin. Nevertheless, it is stated that suckling infants can absorb antitoxin from the mother's milk. Toxin disappears from the blood in the neighborhood of from seven to eleven days after injection, it being in part destroyed, in part bound by the tissues, and in very small part excreted in the urine. Antitoxin appears in man very early in life, as determined by the Schick test (see page 53). It has not been proven why the antitoxin develops, that is, whether it is natural or the result of slight attacks of the disease. As indicated above, it may possibly be transferred in mother's milk. Sherman states that lysins and complement are inappreciable in the youngest swine embryos, but that after the ninth week of gestation they can be demonstrated in varying amount. Whether they are autochthonous or transmitted from the mother has not been determined. Wells states that, "taken together, the evidence indicates a closer resemblance of antitoxins to proteins than has been shown for the toxins, and all attempts to separate antitoxins from proteins have so far failed."

The manner in which antitoxin neutralizes toxin is the subject of much discussion, experiment, and hypothesis. Before discussing the matter from a theoretical point of view, it is advisable to explain some of the technical operations in the standardization or titration of the antitoxin. From the practical point of view this is now relatively simple, although requiring an extremely precise method, but the earlier investigators were beset with many difficulties.

Standardization of Diphtheria Antitoxin.—In diseases such as diphtheria and tetanus, where the symptoms are the results of the action of the toxin, it is necessary to determine the amount of antitoxin required to protect an animal against the effect of a given amount of toxin. The earlier investigators attempted to determine the amount of antitoxic serum necessary to protect against inoculations with living organisms, but the variability in biological properties of growth and toxin production, infection, and resistance, soon showed the unreliability of this method. Behring then took up the determination of antitoxin against toxin, but found it difficult to standardize such a method over a wide geographic area because of

differences in bacterial strains and variations in the same strains growing under even slightly different conditions. Ehrlich sought to reduce the factor of error by determining the antitoxic "unit" as the amount of antitoxic serum necessary to protect against ten times the minimum lethal dose. Even this was unsatisfactory, and Behring and Ehrlich in collaboration settled upon an arbitrary method of determining a "normal" toxin and a "normal" therapeutic serum. The "normal" toxin contained in 1.0 c.c. one hundred times the minimum lethal dose for a guinea-pig of 250 grams. The "normal" therapeutic serum was tested and diluted so that 0.1 c.c. contained sufficient antitoxin to neutralize 1.0 c.c. of the "normal" toxin or, in other words, 1.0 c.c. antitoxic serum, as a unit, was capable of neutralizing 100 minimum lethal doses of toxin. The fundamental error, however, had not been overcome by this method, and it was found that no method which had as its basis a toxin, could be applied over a large area and the method was finally abandoned. The toxin deteriorates rapidly on standing, and even though after a time it becomes fairly stable, it still is insufficiently so to justify its use for purposes of standardization. On the other hand, the antitoxic serum resists drying for an indefinite period, and if used as a standard can be shipped great distances. The standard in this country has been established by the United States Public Health Service. The unit of antitoxin as now used has no direct relation to the unit of "normal" therapeutic serum as defined by Behring and Ehrlich, but by interchange between nations it is practically constant throughout the world. Hence, if a laboratory wishes to prepare an antitoxin the standard unit of antitoxin can be obtained from the Public Health Service. With the standard antitoxin on hand, the antitoxic content of a newly prepared antiserum may be determined. This must be done through the medium of a toxin whose strength is titrated against the standard antitoxin; the toxin is thereby standardized, so that the strength of the new antitoxic serum can be measured. The toxin must be one which has been ripened, so that any deterioration during the few days' time necessary for titration against the standard immune serum and then against the new serum, is reduced to a negligible minimum. In order to make the titration against the standard antitoxic unit somewhat easier it is well to know the minimum lethal dose of toxin.

There are then to be determined:

1. The minimum lethal dose of toxin (M.L.D.).
2. The L_0 dose of antitoxin.
3. The L_1 dose of antitoxin.

The minimum lethal dose of toxin is determined by injecting subcutaneously, varying doses of toxin into a series of guinea-pigs 250 grams in weight. Healthy pigs of this weight are usually young and less expensive than fully-grown animals. The M.L.D. is the smallest dose that kills a pig in from four to five days. Less than four days means too great strength,

more than five days too little strength. It can be seen that the selection of the weight of the pigs and the length of time are arbitrary but universal standards. A strong toxin might give results as follows:

Guinea-pig	Toxin dose	Result
1	0.0036 c.c.	Lives
2	0.0038 c.c.	Dead 6 days
3	0.0040 c.c.	Dead 4 days 8 hours
4	0.0042 c.c.	Dead 3 days 20 hours
5	0.0044 c.c.	Dead 2 days

Guinea-pig No. 3 died at the right time interval and 0.004 is the M.L.D. of this toxin. Experiments with a preliminary series using more widely varying doses of toxin would be necessary before the final experiment could be set up.

The L₅ dose (Limes null) is that amount of toxin which is so thoroughly saturated with one unit of antitoxin that neither local nor general symptoms appear following the injection of the mixture. An experiment follows:

Guinea-pig	Standard antitoxin	Toxin	Result
1	1 unit	0.36 c.c.	No reaction
2	1 unit	0.38 c.c.	No reaction
3	1 unit	0.40 c.c.	Barely visible congestion
4	1 unit	0.42 c.c.	Moderate inflammation
5	1 unit	0.44 c.c.	Distinct inflammation

In this experiment the dose of toxin, 0.40 c.c., given pig No. 3, is the L₅ dose. The note as to reaction refers to the shaven site of injection.

The L₊ dose (Limes death) indicates the smallest amount of toxin which after mixture with one unit of antitoxin will produce death in four-five days. The plus sign is the mark used in English texts to correspond to the cross mark used in German literature to signify death. An experiment follows:

Guinea-pig	Standard antitoxin	Toxin	Result
1	1 unit	0.44 c.c.	Lives
2	1 unit	0.46 c.c.	Dead 6 days
3	1 unit	0.48 c.c.	Dead 4 days
4	1 unit	0.50 c.c.	Dead 3 days
5	1 unit	0.52 c.c.	Dead 2 days

In this experiment 0.48 c.c. given guinea-pig No. 3 is the L₊ dose of toxin.

Titration of Diphtheria Antitoxin.—In the actual titration of an antitoxin as practised to-day there must be at hand a standard antitoxin of known strength as well as a toxin, whose M.L.D. has been at least approximately determined. The antitoxin has been dried in a vacuum and preserved in sealed U-shaped ampoules which contain the antitoxin in one arm and P₂O₅ or some other hygroscopic substance in the other arm, in order to maintain the dryness of the antitoxin. The ampoule is best kept in a light-proof box in the refrigerator. Against this antitoxin the L₊ dose of a toxin is determined, and against this toxin the new antitoxin is titrated. The amount of antitoxin which protects against the L₊ dose for four days is the antitoxin unit of the new serum. In preliminary experiments the antitoxin is roughly titrated in dilutions of 1:100, 1:200, 1:300, and so on. In each case the antitoxin is used in 1.0 c.c. amounts and the toxin so diluted that 2.0 c.c. contain the M.L.D., the two being mixed and allowed to stand at room temperature for

one hour. The Rosenau glass syringe for this purpose has an oblique side arm for salt solution, so that after the toxin-antitoxin mixture is injected, the side arm is swung around, emptying the saline into the main body of the syringe. The salt solution is then injected, thus washing out the remnants of the toxin-antitoxin mixture that may remain in the lower part of the syringe and needle.

The following experiment will serve to illustrate, granting that the preliminary titration showed a strength of antitoxin between 1-200 and 1-400.

Guinea-pig	Antitoxin 1 c.c. of each dilution	Toxin 2.0 c.c. = M.L.D.	Result
1	1-200	2.0 c.c.	Dead 5 days
2	1-220	2.0 c.c.	Dead 5 days
3	1-240	2.0 c.c.	Dead 4 days
4	1-260	2.0 c.c.	Dead 4 days
5	1-280	2.0 c.c.	Dead 3 days
6	1-300	2.0 c.c.	Dead 3 days
7	1-320	2.0 c.c.	Dead 3 days
8	1-340	2.0 c.c.	Dead 3 days
9	1-360	2.0 c.c.	Dead 2 days
10	1-380	2.0 c.c.	Dead 2 days

Thus doses 1 and 2 were more than sufficient to protect four days, and doses 5-10 were insufficient. Doses 3 and 4 protected for four days, and in order to be safe dose No. 3 of 1-240 would be selected. If the antitoxic unit is $1/240$ of 1.0 c.c., each c.c. of serum contains 240 units of antitoxin. In commercial work, the practice is to be absolutely on the safe side, and the next larger dose of antitoxin would be employed as the unit, and the serum marketed as containing 220 units per c.c.

In the therapeutic use of such a serum the unit content of the serum is simply a guide to its use, the dose employed being rather on an empirical basis than otherwise, because of the uncertainty of the amount of toxin present in the body of the patient. It is generally assumed that the larger the extent of the exudate, the greater the amount of toxin produced and the larger the absorbing surface, but it can readily be seen from the theoretical standpoint that variations may be produced by differences in toxin production by the different strains of *bacillus diphtheriæ* which may be encountered in patients. It is unwise to stress this latter possibility and preferable to regulate the dosage on the former basis. More will be said later regarding therapeutics.

The Toxin-antitoxin Union.—The Ehrlich Theory.—With the foregoing practical consideration of antitoxin titration in mind, the theoretical problems of the nature of the toxin-antitoxin combination will be taken up. The simplest conception is that antitoxin neutralizes toxin in the same way that a strong acid neutralizes a strong base. As has been seen, the neutralization is quantitative and follows in a general way



FIG. 2.—The Rosenau or Reichel syringe for injecting toxin-antitoxin mixture.

the law of multiple proportions. If this were true, however, the L_4 dose which in combination with the antitoxin unit kills a pig in four days should contain one unit more of toxin (1 M.L.D.) than the L_0 dose which just fails to produce symptoms. Reference to the experiments offered to illustrate the determination of M.L.D., L_0 dose, and L_4 dose show that the M.L.D. of the toxin was 0.004 c.c., the L_0 dose of toxin was 0.40 c.c. or one hundred times the M.L.D., and the L_4 dose 0.48 c.c. or one hundred and twenty times the M.L.D. The difference between L_0 and L_4 doses is, therefore, twenty times the M.L.D. instead of exactly equal to it. This has been interpreted to indicate that some body or bodies, other than the toxin, has combined with the antitoxin, thus limiting its ability to combine with toxin. Ehrlich, after numerous experiments and hypotheses, reached the assumption that the toxic broth contains two bodies other than toxin, which he named toxon and toxoid. The toxon is a body with a smaller degree of affinity for the antitoxin than has the toxin. In a determination of the L_0 dose the antitoxin neutralizes both toxin and toxon, so that no symptoms appear, but if more toxin be added to the mixture it combines with antitoxin, displacing the more loosely combined toxon. Finally, after sufficient addition of toxin the antitoxin is fully saturated, and any additional toxin will be free, and if in sufficient quantity (1 M.L.D.) will lead to the death of the experimental animal. In more detail the 20 M.L.D.'s necessary to make the difference between the L_0 and L_4 doses were so used that 19 M.L.D.'s were employed to displace a proportionate amount of toxon and toxoid from combination with the antitoxin unit, and the remaining 1 M.L.D. sufficed to kill the pig in four days. If more than 1 M.L.D. were present in excess death would ensue after a shorter period, and if less than 1 M.L.D. were present death would occur later than four days or not at all. It is believed, on the basis both of experimental and clinical observation, that toxon is responsible for the late paralyses of diphtheria. The conception of the toxoid is based on the Ehrlich assumption that the toxin molecule has a toxic fraction or "toxophore group" and a combining fraction or "haptophore group." A toxin will retain its binding power for antitoxin for a considerable length of time with little change in the L_4 dose, but with marked deterioration of toxic power and corresponding reduction of the M.L.D. This is interpreted as meaning that the toxic fraction is labile and the combining fraction much more stable. The toxoid, then, is the toxin molecule so altered that its toxic part is reduced and the combining part practically intact. As can readily be seen, this can account also in part for the discrepancy between L_0 and L_4 dose. The discrepancy between L_0 and L_4 dose in fresh toxic broth is believed to be due to the presence of toxon rather than toxoid, because too short a time has elapsed to account for toxoid formation. As the toxic broth becomes older the discrepancy becomes greater, even after a relative equilibrium has been established, and the differ-

ence is believed to be due to the progressive formation of toxoid from toxin and perhaps also from toxon. Ehrlich and also Madsen found that the combination of one antitoxic unit with toxin in the determination of the L_0 dose was in multiples of 100 M.L.D.'s. These multiples were rarely less than 100 and never more than 200. This would indicate that the multiple is not less than 100, but even though values of 200 are not obtainable, the failure may be explained by the fact that pure toxin is not procurable. By means of the phenomenon of "partial absorption" Ehrlich established a formula for the antitoxin-toxin combination which he expressed as "toxin₂₀₀ antitoxin." This has been illustrated by means of a toxin-antitoxin "spectrum" based on a total valency of 200, the total valency including toxin, protoxoid, and toxon. In spite of the great academic interest of this discussion, its immediate practical value is not apparent and the reader is referred to larger works for complete discussion.

Objections to the Ehrlich Theory.—As indicated previously, the Ehrlich hypothesis is based on the assumption that the toxin-antitoxin reaction follows in a fairly close manner the chemical reaction between a strong acid and a strong base. Certain features of the process of combination support the idea of chemical union, as, for example, the fact that warmth accelerates the reaction, dilution slows it. Furthermore, there is a liberation of heat in the reaction, that is to say, about half as much heat per gram molecule as would be liberated by the reaction between a strong acid and a strong base. It is well known, however, that the union of toxin and antitoxin is loose and within certain limits reversible. The Martin and Cherry experiment referred to earlier in this chapter is of great importance in this connection. They mixed snake venom and antitoxin to a point of neutralization and filtered through gelatin filters, with the result that the toxin came through the filter first. This they interpreted as being due to the smaller size of the toxin molecule. It also shows the looseness of combination and the reversibility of the reaction. They further showed that the longer the mixture stands, the smaller the amount of toxin that comes through the filter. Zinsser states that the "chief value of these experiments lies in their proof of the element of time as an important factor in the toxin-antitoxin union." Calmette had previously shown that venoms of certain snakes would remain virulent after heating even to 100 degrees, and that the antitoxins were thermolabile. He demonstrated that if the two were mixed so as to be non-toxic, subsequent heating would liberate the toxin probably through thermic destruction of the antitoxin. If the union were a fixed one, this should not have been true. Martin and Cherry failed to confirm this with the venom of an Australian snake, but this cannot be regarded as a refutation of Calmette's work, especially as the principle was found to apply to other toxins and antitoxins. Morgenroth showed that acidulation with HCl of a venom lysin-antilysin mixture produced an acid-toxin molecule that resisted

heat and could by heat be dissociated from the thermolabile antilysin. The subsequent chemical neutralization of the toxin (or lysin) resulted in the restoration of its toxicity. In this laboratory Wahl has shown that titration of diphtheria toxin using normal guinea-pigs in one series and guinea-pigs with only one kidney in another, gives materially different results. These experiments were carefully controlled and may be offered as a further indication of the loose combination and its corollary the reversibility of the reaction, on the probable assumption that the toxin is more readily excreted by the animals with two kidneys than by those with one. The recitation of these few experiments to which others might be added is sufficient to illustrate the objection to the Ehrlich theory of fixed combination, and two other important hypotheses are offered for consideration: (1) The conception that the combination follows the law of mass action and (2) the theory of colloidal reaction.

The Law of Mass Action.—The application of the law of mass action has been worked on by Arrhenius and Madsen principally. This law is usually illustrated in the chemical laboratories by the reaction between one gram molecule ethyl alcohol and one gram molecule acetic acid which yields ethyl acetate and water, the reaction, however, stopping at a point of equilibrium where there is found in the mixture $\frac{1}{3}$ gram molecule alcohol, $\frac{1}{3}$ gram molecule acetic acid, $\frac{2}{3}$ gram molecule ethyl acetate, and $\frac{2}{3}$ gram molecule water. The same end-result is obtained if instead of mixing ethyl alcohol and acetic acid, we mix ethyl acetate and water, thus indicating the reversibility of the reaction as stated in the formula:



Arrhenius and Madsen compared the reaction between tetanolysin and its antitoxin and the reaction between boric acid and ammonia. This was of advantage because ammonia is hemolytic and boric acid is not. Thus a reversible reaction is found in which the addition of boric acid reduces the hemolytic activity of the ammonia. As with the alcohol-acetic acid experiment, however, a point of equilibrium is established whereby there always remains a small amount of free ammonia in spite of the addition of boric acid to a point of saturation. The same general proposition holds in regard to tetanolysin and antilysin, and these authors were able to construct similar curves of neutralization for both of these reactions. With this idea as a basis, the late paralyzes of diphtheria, either experimental or clinical, would depend not upon the toxon of Ehrlich, but rather upon a small non-fatal amount of toxin that is never completely neutralized in the reaction.

The Danysz Effect.—It will readily be seen, however, that the reversible reactions illustrating the law of mass action deal with crystalloids, while it is probable both toxins and antitoxins are of colloidal character. Certain colloids are known as "reversible col-

loids," but as yet there is little definite proof that reversible reactions between two colloids take place. According to certain interpretations, the most important observation in support of the colloidal theory is the Danysz effect. If the toxin is added to the antitoxin in fractions with an interval of time elapsing between, less toxin is needed to saturate the antitoxin than if the toxin were added in one volume. In other words, if 1.0 c.c. toxin were saturated in the usual way with 0.1 c.c. antitoxin and if in another test-tube the toxin is added to the same amount of antitoxin, not in a single dose, but in successive doses of 0.2 c.c., until 1.0 c.c. is present, this latter mixture instead of being neutral would be toxic. Wells, however, states that this "indicates that the toxin antitoxin union is physical rather than chemical, for it seems to be quite analogous to such a phenomenon as the taking up of more dye by several pieces of blotting paper added in series to a dye solution, than by the same amount of paper added in one piece." Of somewhat similar import is the absorption theory of Bordet and of Landsteiner, which states that when toxin is added to antitoxin in smaller quantities than saturation, let us say five molecules of antitoxin to ten of toxin, this does not result in complete molecular combination with five molecules of toxin, but rather in half saturation of the entire ten molecules of toxin. This results in attenuation of the toxin, so that instead of there being five free molecules of toxin there are ten units of partly detoxified toxin. It is not to be expected that this follows in exact arithmetical progression, but Biltz has made comparisons with absorption phenomena in general and finds fairly consistent results.

In summary it may be said that in explaining the union of toxin and antitoxin the Ehrlich hypothesis does not withstand critical examination and that the reaction is in all likelihood of an intricate physico-chemical nature referable, in part at least, to the probable colloidal nature of the reacting bodies, but not as yet satisfactorily explained.

Therapeutic Use of Diphtheria Antitoxin.—In 1892 von Behring and Wernicke found that the serum of animals immunized against diphtheria toxin protects other animals of the same and different species against the action of the toxin. In 1894 Roux demonstrated the value of the treatment of diphtheria in man by means of antitoxic serum. This method of treatment rapidly attained widespread use and has markedly reduced the mortality from the disease. Numerous statistical studies have been made since that time, and it is safe to say that the introduction of antitoxin treatment has reduced mortality from approximately 40 per cent. to approximately 7 per cent. In interpreting the figures it has been found necessary to take account of two important factors; namely, the cases of laryngeal diphtheria and the time at which treatment is instituted. Laryngeal diphtheria presents not only the element of toxic absorption, but in addition mechanical obstruction to respiration and the possibility of extension downward, so as to produce pneumonia. Furthermore, the operative procedures for relieving the respiratory

obstruction are such as to introduce an additional minor element of danger. The accidents following tracheotomy are distinctly more numerous than those following intubation, but neither operation can be regarded as absolutely without risk. It is now well established that the earlier in the course of disease the antitoxin is administered the more favorable is the prognosis. In order to present this graphically, however, we insert the following table taken in large part from Dieudonné and Weichardt:

	Total number of cases	Mortal- ity per- centage	1st day	2nd day	3rd day	4th day	5th day	6th day	After 6th day
Welch.....	1489	14.2	2.3	8.1	13.5	19.0	29.3	34.1	33.7
Hilbert.....	2428	18.3	2.2	7.6	17.1	23.8	33.9	34.1	38.2
American Pediatric Society.....	5794	12.3	4.99	7.4	8.8	20.7	35.3		
Brook Hospital, London.....	8097	9.5	0.0	4.3	11.12	17.24	18.72		
<i>Germany</i>									
Kais-Gesundh. Amb- tes (Berlin) (Sam- melforschung)....	9581	15.5	6.6	8.3	12.9	17.0	23.2	26.9	
<i>Russia</i>									
(Rauchfusz) (Sam- melforschung)....	44,631	14.6	3.7	8.2	16.2	25.9			
<i>Austria</i>									
Sammelforschung (Sanitätswesen)...	1103	12.6	8.0	6.6	9.8	25.5	28.8	30.7	21.0

The therapeutic efficiency of the antitoxin also varies according to the method of administration. According to Berghaus, intravenous injections are five hundred times more effective and intraperitoneal are eighty to ninety times more effective than subcutaneous injections. In the earlier days of antitoxin treatment the method was almost entirely subcutaneous injection, but subsequently the intravenous method was employed in severely toxic cases. Injections were given at various intervals, usually a day apart, until the disease showed marked improvement. The studies of Park and his collaborators have modified the treatment considerably. Park was able to show that a single dose of antitoxin in sufficient quantity is more effective in neutralizing the circulating toxin than the multiple small doses, largely because of the fact that with subcutaneous and intramuscular injections the absorption is continuous, whereas during the period usually occupied by giving several doses, the absorption occurs for only a short time after each injection. Were it possible to determine for clinical purposes the exact amount of toxin absorption during the disease the dosage of antitoxin could be accurately regulated. Unfortunately, however, different strains of the bacilli vary in capacity for production of toxin and the depth and extent of the local lesion, as well as the nature of the underlying tissues, have some influence upon the rate and amount of absorption.

Therefore, the actual dose employed is to a large extent upon an empirical basis. Park recommends the following table of doses and methods of administration :

DOSAGE OF UNITS OF ANTITOXIN IN DIPHTHERIA. SINGLE DOSE ONLY.

Infant, ten to thirty pounds (under two years of age).

Mild	Moderate	Severe	Malignant
2,000	3,000	5,000
3,000	5,000	10,000	10,000

Child, thirty to ninety pounds (under fifteen years of age).

3,000	4,000	10,000	10,000
4,000	10,000	15,000	20,000

Adults, ninety pounds and over.

3,000	5,000	10,000	15,000
5,000	10,000	20,000	40,000

METHOD OF ADMINISTRATION.

Mild	Moderate	Severe	Malignant
Subcutaneous or intramuscular	Intramuscular or subcutaneous	Intramuscular or $\frac{1}{2}$ intravenous and $\frac{1}{2}$ intramuscular or subcutaneous	$\frac{1}{2}$ intravenous and $\frac{1}{2}$ intramuscular or subcutaneous

McCombie recommends the following dosages:

Mild: 4000 to 8000 units in one dose.

Moderate: 12,000 to 16,000 units in one dose or two doses.

Severe: 20,000 to 50,000 units or more in two or three doses.

Laryngeal: 16,000 to 24,000 as initial dose, and repeat once or twice according to persistence of symptoms.

Improvement following the administration of antitoxin is striking when given early and exhibits itself in fall of temperature, reduction of leucocytosis, reduction of inflammation, and separation of the fibrinous membrane. This improvement varies somewhat with the method of administration, the intravenous method effecting improvement in somewhat less time than the intramuscular, and the latter in somewhat less time than the subcutaneous. In a series of cases studied in the City Hospital in Cleveland by Ruh the intravenous form of administration was followed in 83 per cent. of the cases by a severe general reaction with chills and prostration coming on a few minutes after the administration and lasting for about twenty minutes. At the present time it is impossible to state the exact cause of this reaction. Such reaction does not follow subcutaneous and intramuscular injections. It is not due to the preservative, nor as far as can be determined, to the age of the serum. The most reasonable explanation appears to us to be that the reaction is due to foreignness of the horse protein. None of Ruh's cases showed prolonged or fatal reactions, and it is not probable that these reactions represent individual hypersusceptibility, because if this were true fatalities would be likely to occur (see page 230).

Natural Immunity to Diphtheria—The Schick Test.—It has long been known that many individuals, even as many as 80 per cent. of adults and 50 per cent. of children, are immune to diphtheria, as indi-

cated by demonstrating antitoxin in the blood. The methods of demonstration were not easily applicable until the development of the Schick test. This test is performed by injecting intracutaneously one-fiftieth of the minimum lethal dose of a specially prepared toxin contained in 0.2 c.c. of salt solution. Injection is preferably on the flexor surface of the arm or forearm. Six-day broth cultures of the organism are killed with phenol, sedimented in the ice-box for two or three days, the supernatant fluid filtered through a Berkefeld candle, and the clear filtrate accurately standardized. It is well to keep this filtrate for several months or a year, so that its rate of deterioration is reduced to a minimum. A control injection is given with the same quantity of toxic broth heated to 75° C., so as to destroy the toxin.

The reactions to injections may be as follows:

A. Positive reaction. This indicates that no antitoxin is present in the body, thereby permitting the toxin to act upon the unprotected cells. Slight reaction appears in from twelve to twenty-four hours in the form of redness, which becomes more distinct in from twenty-four to forty-eight hours, reaching its maximum on the third or fourth day, then gradually disappearing and leaving an area of scaling and brown pigmentation. The area attains a diameter of 10 to 20 mm., and varies in intensity, depending on the sensitiveness of the individual.

B. Negative reaction. If no distinct reaction appears any more than is seen in the control area the failure to react indicates that an amount of antitoxin is present in the body sufficient to neutralize the introduced toxin. Such a reaction in a child of about three years of age probably indicates permanent immunity. By varying the quantity of toxin injected, the amount of antitoxin can be titrated.

C. The pseudo-reaction. This is usually urticarial in nature, appearing sometimes immediately and sometimes in from six to eighteen hours, reaching its maximum on the third or fourth day. It fails to leave pigmentation after it subsides. This is a reaction of hypersusceptibility to the protein substances present in the toxic broth as the result of the autolysis of the diphtheria bacilli, and is in nature the same as other reactions of hypersusceptibility described subsequently (see page 236). Such a pseudo-reaction may intensify the true reaction and represent a summation of the protein reaction and a reaction to the toxin. This must be taken to indicate that an individual may be hypersensitive to the proteins of diphtheria bacilli but at the same time not possessing in his circulating blood any antitoxin. The differentiation depends upon the difference between the reaction at the site of the test injection and at the site of the control injection.

Zingher divides the positive reactions as follows: ++ indicates a strong positive reaction with marked local redness, infiltration, and occasionally superficial vesiculation; + indicates positive reaction with redness but little or no infiltration; ± indicates moderately positive reaction with moderate degree of redness and no local infiltration; ± indicates a faintly positive reaction with only slight redness and no local infiltration.

The test has been found to be of great value in determining the immunity of groups of individuals, particularly in institutions where there has been exposure to diphtheria. It has also given considerable information as to the incidence and duration of this variety of active immunity. Immunity to diphtheria may be derived from the mother and lasts for about six months after birth. The largest number of positives is found from the ages of six to eighteen months. This gradually decreases throughout life.

Another method for determining the presence of antitoxin in the blood is that of Römer. This depends upon the well-known fact

PLATE I.



E. F. Fisher

POSITIVE SCHICK REACTION

Reaction of moderate severity seventy-two hours after the intracutaneous injection of one-fortieth the minimal lethal dose of diphtheria toxin. Patient's blood serum was found to contain no antitoxin (International Clinics).

that the intracutaneous injection of toxin into guinea-pigs leads to localized necrosis in the course of forty-eight hours. The minimum amount of toxin sufficient to produce necrosis can be determined, the protective power of antitoxin determined, and subsequently with a standard antitoxin any new toxin may be titrated after the same general principles as described previously for antitoxin titration. By this method the presence of toxin in human blood may be determined, inasmuch as normal human serum does not produce necrosis upon intracutaneous injection in the guinea-pig. Harriehausen and Wirth found that the serum from patients suffering with diphtheria produced necrosis owing to the presence of toxin, and this was demonstrated in five cases for as long as thirty-five days after the onset of the disease. By the use of a titrated toxin the method may also be employed for determining the presence and amount of antitoxin in human blood.

Active Immunization Against Diphtheria.—For many years immunization to this disease was entirely in the form of passive immunization, practised by giving protective doses of antitoxin. The antitoxin was given in doses of 500 to 1000 units and served to protect for a period of about three to six weeks. This was of special importance in institutions and families exposed to the disease. The disadvantages are the short period of immunity and the fact that the patient may thereby become hypersensitive to any subsequent injections of horse serum. Active immunity had been observed by Park in guinea-pigs which had been used for the titration of antitoxin, and Park, in 1905, reported that horses treated with neutralized mixtures of toxin and antitoxin had produced immune sera as strong as 400 units per c.c. Theobald Smith suggested a similar method of immunization in man, and in 1913 von Behring reported the successful immunization of children and adults. The method of immunization with toxin and antitoxin mixtures has now attained a widespread use and is employed even as early as the fourth day of life. Active immunity of this sort is demonstrable by the Schick test in about ten days after treatment, and increases so that in the eighth week about 80 per cent. of the treated individuals are immune, by the twelfth week 96 per cent. are immune, and at the end of four months 98 per cent. are immune. According to Park, the remaining 2 per cent. become immune if reinjected. The method of immunization is to give three injections subcutaneously one week apart.

For the preparation of the mixture a ripe toxin is used and so diluted that 1. c.c. will contain 200 minimum lethal doses as tested against guinea-pigs. This is slightly overneutralized with antitoxin and the mixture should cause no symptoms in guinea-pigs even when given in very large doses. As indicated above, antitoxin may deteriorate in the moist state, and this must be avoided in the toxin-antitoxin mixtures. If the mixtures are kept at about 21° C. the mixture remains good for at least one year, although it is preferable to keep it at a lower temperature. The injection is given subcutaneously in the arm at the insertion of the deltoid muscle. The immunity developed following injection of this sort is against toxin, but vaccination against diph-

theria bacilli themselves may also be practised at the same time by adding 1000 millions killed organisms. The value of this latter procedure has not been demonstrated as yet and it is not widely practised.

The advantage of the use of toxin-antitoxin mixtures is that a lasting active immunity is established. It has been suggested, however, that the use of the antitoxin in the mixture may lead to the development of anaphylaxis. It has been maintained, on the other hand, that the mixture of the toxin probably avoids the sensitizing effect of the horse serum, but this is not borne out experimentally or in human medicine. Nevertheless, the amount of horse serum given is relatively small and the development of hypersusceptibility in man following these small amounts is not very common. Park has found that even with the therapeutic dose of diphtheria antitoxin the danger of anaphylaxis is extremely small, and states that among 330,000 cases on record there were only five deaths. As we will point out subsequently (see page 230), the reports of deaths following antitoxin administration would indicate that, in a certain percentage at least, factors other than anaphylaxis are operative. If sensitiveness to horse serum is known it is suggested that antitoxin prepared in some other animal, such as the goat, may be employed, but sera of this sort are not easily obtainable in the market.

Tetanus Toxin and Antitoxin.—In the foregoing consideration much stress has been laid on diphtheria toxin and antitoxin, with the idea that the problem might thus be presented as simply as possible. Tetanus toxin and antitoxin have been studied almost if not quite as intensively as diphtheria and deserve, both practically and theoretically, more than passing mention. The important facts may be given briefly. The toxic broth produced by growth of *bacillus tetani* contains a body which is actively hemolytic, *tetanolysin*, and more important, a body which produces the symptoms of tetanus, *tetanospasmin*. These are capable of specific absorption, so that one or the other remains free. In other words, the tetanolysin may be removed from the mixture by saturation with red blood-corpuscles, leaving in the broth the tetanospasmin which may be injected with the production of symptoms and death. An antitoxin may be produced specifically for the tetanospasmin, but in practice the antitoxin is made without separation of the two toxins.

The toxin is produced in anaërobic broth cultures. It is readily injured by heat and light, and is best preserved in the dried state. The white mouse is extremely susceptible, the guinea-pig less so and the horse somewhat less, whilst pigeons and fowl are highly resistant. The antitoxin is produced for commercial purposes in the horse, the earlier doses being with an attenuated toxin following a previous injection of antitoxin. The serum is standardized by the use of white mice or by guinea-pigs, the procedure being practically the same as for standardization of diphtheria antitoxin. In the United States the toxin is used as a standard. It is precipitated by ammonium sulphate and dried. The minimum lethal dose is that

which kills a pig of 350 grams in four to five days. The antitoxic unit is ten times the amount of antitoxin necessary to protect against 100 minimum lethal doses.

In man the symptoms appear, as a rule, first in trismus of the jaw muscles, but in experimental animals the first spasms are near the injection site when the toxin has been given subcutaneously or intramuscularly. For demonstration purposes the dried toxin is freshly dissolved and five minimum lethal doses injected into the thigh muscles of one hind leg of a guinea-pig. In the course of about two days the leg is found stiff and extended, the animal showing excitable reflexes. In the course of another day a sudden noise or other stimulus will excite convulsions, and later the animal will be found in tonic spasm and dies with all four extremities in extension. If the toxin is given intravenously or intraperitoneally, the first symptoms are excitable reflexes, then general clonic and finally tonic spasm. If given intracerebrally, the onset is by epileptiform convulsions. Rabbits are much more resistant to the toxin, and an intravenous injection will lead to gradual wasting and a cachectic death, which has been called "tetanus sine tetano." The susceptibility of animals varies with the temperature of the body. Cold-blooded and hibernating animals are resistant at cold winter temperatures, but become susceptible at summer temperatures.

Tetanolysin. The tetanolysin is easily demonstrable in a toxin. The best red blood-cells for use are those of the goat, sheep and horse. The following protocol will show the method of titrating the tetanolysin. The toxin is dissolved so as to make a 1 per cent. solution in saline, and is further diluted for the experiment 1-2, 1-5, 1-10, 1-20. The blood-cells are washed three times and suspended in salt solution so as to make a 5 per cent. solution. For method of washing red blood-cells see page 118.

Tube	Toxin	5% sheep cells	Hemolysis
1	1-2 (1.0 c.c.)	1.0 c.c.	Complete
2	1-5 (1.0 c.c.)	1.0 c.c.	Complete
3	1-10 (1.0 c.c.)	1.0 c.c.	Partial
4	1-20 (1.0 c.c.)	1.0 c.c.	Slight
5	Saline (1.0 c.c.)	1.0 c.c.	None

The mixtures are incubated in a water bath at 37° for one hour.

The minimum lytic dose in the above instance is 1 c.c. of a 1-5 dilution of the 1 per cent. toxin solution. This is used as the unit to determine the antitetanolysin in an antitetanic horse serum as in the following protocol:

Tube	Toxin	Immune serum	5% sheep cells*	Hemolysis
1	1.0 c.c. 1-5 dil.	1-1,000 (1.0 c.c.)	1.0 c.c.	None.
2	1.0 c.c. 1-5 dil.	1-2,000 (1.0 c.c.)	1.0 c.c.	None
3	1.0 c.c. 1-5 dil.	1-10,000 (1.0 c.c.)	1.0 c.c.	Slight
4	1.0 c.c. 1-5 dil.	1-20,000 (1.0 c.c.)	1.0 c.c.	Partial
5	1.0 c.c. 1-5 dil.	1-50,000 (1.0 c.c.)	1.0 c.c.	Complete
Normal horse serum				
6	1.0 c.c. 1-5 dil.	1-100 (1.0 c.c.)	1.0 c.c.	Complete
7	1.0 c.c. 1-5 dil.	1-1,000 (1.0 c.c.)	1.0 c.c.	Complete
8	1.0 c.c. 1-5 dil.	None	1.0 c.c.	Complete
9	None	1-100 (1.0 c.c.)	1.0 c.c.	None

*Add the sheep cells after the mixture of toxin and serum has been incubated for one-half hour and then incubate one hour. For method of diluting serum so as to obtain required strengths see page 84.

Tubes 6-9 are controls to show that normal horse serum is not antilytic, that the laking dose still operates after the preliminary half-hour incubation, and that horse serum itself has no lytic effect.

Tetanospasmin. For the demonstration of the neutralization of tetanospasmin by antitoxin and by brain substance, the following experiments are of value. Five guinea-pigs of about 250 grams are needed.

Pig No. 1. Inject five minimum lethal doses of toxin into the thigh muscles.

Pig No. 2. Mix ten minimum lethal doses of toxin with one unit of antitoxin. Allow to stand at room temperature for about twenty minutes and inject as in pig No. 1.

Pig No. 3. In a sterile mortar grind one-half the fresh cerebrum of a guinea-pig with five minimum lethal doses of toxin, adding salt solution in the smallest amount necessary. Allow to stand two hours, centrifuge and inject the supernatant fluid as in pig No. 1.

Pig No. 4. The other half of a guinea-pig brain is boiled for twenty minutes in water, then ground up with five minimum lethal doses of toxin, allowed to stand two hours, centrifuged, and the supernatant fluid injected as in pig No. 1.

Pig No. 5 serves as a control.

The guinea-pig injected with toxin will show typical symptoms as described above, beginning with extension of the leg injected, then showing excitable reflexes followed by convulsions, tetanic spasm and death. The antitoxin and fresh brain substance will protect the animals, but the boiled brain will not. The normal animal serves best as a control for the elicitation of excitable reflexes and slight convulsions.

Route of Absorption of Toxin.—It is of interest to note that in man, horse, and guinea-pig the central nervous system alone has the power of neutralizing tetanus toxin, but in the case of the rabbit, liver and spleen in addition have this power. It is maintained that the gray substance of the nervous system possesses this special affinity, and the white matter does not. Most authorities believe that the toxin is carried along nerve tracks, but Zupnik maintains that it travels through the blood stream and is found not only in the nervous system, but also in the muscles. Studies of Meyer and Ranşom and of Marie and Teale indicate that both routes are followed. Depending on the size of the dose, the site of inoculation, and perhaps certain other factors, one or the other route may be followed predominantly, but never to the exclusion of the other. According to Teale and Embleton, the mode of transit along nerve trunks is by way of the axis cylinders and the perineural lymphatic vessels. These authors, however, maintain that toxin cannot pass from the choroidal plexis into the cerebrospinal fluid, nor from the capillaries of the central nervous system to the nerve tissues. The special affinity of tetanospasmin for nerve substance is not peculiar and is also exhibited by the neurotoxins of snake venom and by the toxin of bacillus botulinus. Teale and Embleton believe that tetanus antitoxin does not enter the substance of the central nervous system following either intravenous or intrathecal injection, but simply acts by neutralization of the toxin at the site of formation. Clinical experience is not entirely in agreement with the experimental work of these authors, since cases have been improved by the use of serum after tetanic spasms have appeared.

Therapeutic Use of Tetanus Antitoxin.—As is well known, tetanus

follows the introduction of the bacilli or their spores into wounds in such a fashion that anaërobic growth is permitted. The incubation period is usually considered to be eight days, but there are many variations from this standard period, including cases that have an incubation period of over sixty days. The mortality from the disease is extremely high, the average ranging between 78 and 90 per cent. Its incidence in civil life is not very great, but in time of war it is likely to occur with considerable frequency because of the contamination of war wounds by soil containing the organism or its spores. In the American Civil War the disease occurred in 2.5 per cent. of the wounded; in the Franco-Prussian War in 3.5 per cent.; and in the World War 6.5 per cent. In the earlier wars the mortality ranged between 80 and 90 per cent., but in the World War, owing in all probability to prophylaxis and treatment, the mortality was 50 per cent. In carefully studied statistics it is found that the longer the incubation period the lower is the rate of mortality. This general statement held true before the use of anti-tetanic serum was instituted and still holds true. The difference between the mortality rate of 80 to 90 per cent. in the earlier wars and 50 per cent. in the World War gives an excellent illustration of the decrease in mortality that has followed the introduction of serum prophylaxis and treatment as well as rational surgery. Knowing that the organism is anaërobic in growth, surgery demands that contaminated wounds be kept open for the access of air.

Prophylactic Use of Serum.—The use of tetanus antitoxin is directed toward prophylaxis and toward cure. As can readily be understood from the experiments outlined above, the toxin of this disease is very firmly bound to nerve tissues; therefore, treatment established after the disease has appeared is not likely to be so effective as in the use of some other antitoxins. Nevertheless, notable success has been attained in some cases where the disease has become well advanced before serum treatment has been instituted. Prophylactic treatment with serum is given as early after the wound as possible, and in both military and civil life all wounds contaminated with soil should receive protective doses of tetanus antitoxin. This is given subcutaneously in doses of 500 to 1000 units. Wolff reported that in the German army prior to December, 1914, prophylactic injections were not regularly given and the incidence of tetanus amounted to 1.4 per cent. of the wounded. During the following seven months prophylactic injections were given in the field to all those wounded by grenades and shrapnel, but not those wounded by rifle bullets, and the incidence of the disease was reduced to 0.16 per cent. Protection was equally as successful in the Allied armies, and instructions were given to administer serum as soon after injury as possible, either in the first-aid station or in the field hospitals. Experiences in the British army demonstrated that cases might develop a considerable time after the wound was inflicted, and for this reason subsequent orders directed the use of

500 units of tetanus antitoxin every ten days for four doses. It was further recommended that the serum be given subcutaneously not more than seven days and not less than two days before any operative procedure upon an old wound. If haste is necessary the serum may be given intramuscularly twelve hours before operation. Obviously this suggests the possibility that organisms may remain dormant in wounds, to become active at a later period; it is further believed that the antitoxin is probably eliminated in about ten days, and the later doses of immune serum are given in order to neutralize any toxin that might be produced subsequently.

Golla tabulated the following cases not receiving prophylactic doses of antitoxin:

Incubation period	Cases	Mortality
1-7 days	17 (32.7 per cent.)	82.5 per cent.
8-14 days	24 (46.2 per cent.)	79.0 per cent.
15-21 days	6 (11.5 per cent.)	54.0 per cent.
Over 21 days	5 (9.6 per cent.)

This shows that the commonest incubation period is eight to fourteen days, and also illustrates the fact that the shorter the incubation period the more serious is the disease. In another series of patients who had received prophylactic treatment with serum, the following data were collected:

Incubation period	Cases	Mortality
1-7 days	61 (22.6 per cent.)	75.5 per cent.
8-14 days	93 (34.6 per cent.)	70.0 per cent.
15-21 days	33 (12.2 per cent.)	60.8 per cent.
21-30 days	19 (7.05 per cent.)	62.8 per cent.
30-40 days	14 (5.2 per cent.)	57.0 per cent.
40-50 days	9 (3.3 per cent.)	33.4 per cent.
50-60 days	18 (6.7 per cent.)	27.7 per cent.
Over 60 days	22 (8.2 per cent.)	40.8 per cent.

In the Franco-Prussian War only 5.7 per cent. exhibited an incubation period of more than twenty-one days, whereas, according to Golla, in the last war 30.54 per cent. showed an incubation period of more than twenty-one days. In summary it may be stated that the introduction of prophylactic injections of the tetanus antitoxin not only reduces the incidence of the disease, but also lengthens the incubation period, and therefore reduces the mortality. The delay in incubation usually leads to more moderate symptoms as well as reduces mortality, and oftentimes the cases exhibit tetanic spasms in only one extremity.

Treatment of Tetanus with Serum.—When the disease has developed, treatment must be prosecuted vigorously. In the earlier years of its employment subcutaneous, intramuscular, and intravenous administration was practised, but arguing from the nature of the disease it was soon suggested that intrathecal injections be given. This suggestion was followed by experimental and clinical investigation, and in the hands of the majority of workers the method has been found to have great value. Park and Nicoll injected twice the

fatal dose of toxin into the hind legs of guinea-pigs and seventeen to twenty-four hours subsequently injected antitoxin by various routes. Six animals receiving the immune serum subcutaneously died; fifteen received it intracardially and two survived, whereas sixteen animals received it intrathecally and thirteen survived. It was found that the dose necessary for intrathecal injection was considerably smaller than the dose necessary for injection into the circulation. Sherrington conducted a similar series of experiments upon monkeys with essentially the same results. He used twenty-five monkeys for his series of injections and the following table gives the results:

Route of injection	Time between giving of toxin and antitoxin	Recoveries	Deaths
Lumbar intrathecal	47-78 hours	14	11
Bulbar intrathecal	47-78 hours	13	12
Intravenous	47-78 hours	7	18
Intramuscular	47-78 hours	3	22
Subcutaneous	47-78 hours	2	23
Cerebral subdural, ten cases	47-78 hours	0	10

Clinically Irons was not able to demonstrate such a marked difference in results, and Leishman and Smallman came to the conclusion that the intramuscular route is the best. The work of Andrew and Golla demonstrated the clinical value of the intrathecal method. Experimental work also shows that although antitoxin can take up toxin after fixation with nerve tissue, such a release of toxin is restricted by long contact with the nerve tissue. This explains the necessity for early administration of antitoxin. For example, the experiments of Doenitz show that the amount of antitoxin necessary for protection increases tremendously with a lapse of time. He injected twelve times the fatal doses of toxin and found that after the lapse of four minutes a slight excess of antitoxin was sufficient to protect the animal, but after eight minutes six times this dose of antitoxin was required; after sixteen minutes twelve times the dose, after 1 hour twenty-four times the dose; in four to six hours six hundred times the original dose, and after six hours he was unable to save the animals. As a result of long experience with treatment it has finally been determined that a combination of modes of injection is desirable in order to procure complete and lasting saturation of the body with antitoxin. When giving intrathecal injections it is well to draw off the spinal fluid and then immediately inject 3000 to 5000 units of toxin, diluting the serum to a volume of 10 to 15 c.c. with sterile salt solution. Where no fluid can be withdrawn from the spinal canal the antitoxin is introduced very slowly by gravity. The intrathecal injection is further supplemented by 10,000 to 15,000 units intravenously, and three to four days later a similar injection subcutaneously. It is often advisable to repeat the intrathecal injections each day for three or four days. The following outline taken from the Memorandum

on Tetanus published by the British War Office gives a plan for combined injections in a case of acute tetanus:

Day	Subcutaneous	Intramuscular	Intrathecal
First	8,000 units	16,000 units
Second	8,000 units	16,000 units
Third	4,000 units	8,000 units
Fourth	4,000 units	8,000 units
Fifth	2,000 units
Seventh	2,000 units
Ninth	2,000 units

This outline is offered as a suggestion for treatment and has been applied successfully. The doses are arranged in multiples of 8000 because that was the size phial issued in the British army. Doses may be varied, but it is strongly advised to administer a total of 75,000 to 100,000 units. In those cases with long incubation period the dose may be smaller, and if the case is one of spasm in one extremity, without evidence of involvement of higher centers, such as spasm of jaw muscles (trismus), the serum may be given by intramuscular and subcutaneous routes in amounts of 3000 to 6000 units. The patient should be placed so that he lies with the feet considerably higher than the head, so as to allow drainage toward the head. It has also been suggested that the antitoxin be applied near or in the wound. Calmette recommended that powdered antitoxic serum be applied locally. Suter recommended rubbing the fluid serum into the wound. Bockenheimer recommended that it be introduced in the form of ointment, and Robertson saturated pads of cotton with antitoxin, dried these for twenty-four hours at 40° to 45° C., and applied them locally. As will be seen, these latter measures are probably more in the nature of prophylaxis than treatment, and no definite information has accrued as to their value. The disadvantages of serum treatment are essentially the same as those in the use of diphtheria antitoxin, but in addition we have to deal with the factor of introduction of foreign serum into the spinal canal. This frequently leads to the development of a sterile meningitis with a formation of purulent fluid. As far as can be learned, this inflammation does no damage. A few reports of nerve and cord lesions following the use of antitetanus serum intrathecally have been reported, but they are extremely small in number compared to the number of cases treated, and it would appear that the high percentage of mortality in this disease justifies the intrathecal treatment in spite of the minor element of danger.

Dysentery Toxin and Antitoxin.—Dysentery toxin may be produced in broth by the growth of the Shiga bacillus. It is probable that the Flexner and Hiss-Russell types produce only an endotoxin. This is consistent with the greater clinical and pathological severity of the Shiga type of dysentery. The broth must be definitely alkaline, the optimum stated by Doerr being reached where 0.3 per cent. soda is added to a broth neutral to litmus. Rabbits are very susceptible and the intravenous injection of a filtered toxin broth in

proper doses will produce marked, often bloody, diarrhea, wasting, paresis, or even paralysis of extremities, and death. The autopsy shows marked inflammation, often hemorrhagic, particularly severe in the cecum, but also involving the large intestine and lower ileum. Monkeys, cats, and dogs are also susceptible, but fowl, pigeons, and guinea-pigs are resistant. Antitoxin can be produced in horses and goats. There is considerable difficulty in standardizing such a serum, owing to the variation in individual susceptibility of animals. Kraus and Doerr have shown that the immune serum first shows a capacity to neutralize toxin *in vitro*, then *in vivo* (simultaneous injection of toxin and antitoxin into opposite ear veins), and finally it attains a definite curative value as demonstrated by primary injections of toxin followed after certain time intervals by antitoxin. A serum must have a high curative value before it is acceptable and is used in doses of cubic centimeters rather than units.

Therapeutic Use of Anti-dysentery Sera.—After the discovery of the dysentery bacillus by Shiga in 1898 it was found that the separate types of this organism vary greatly in their power to produce toxic substances. The most toxic varieties are those of Shiga and Kruse, and their toxins are not only endotoxic but also exotoxic in nature, a fact clearly established by the work of Todd, Lüdke, Kraus, Doerr, and Rosenthal. Shiga was the first to immunize horses with killed cultures of his organism and produced highly protective sera capable of saving guinea-pigs injected with six times the lethal dose of living bacilli. This specific anti-bacterial serum was used by Shiga with encouraging results during a dysentery epidemic in Japan, the mortality among cases treated with Shiga's serum being one-third of that among cases treated by the usual routine procedures. Not only was the mortality greatly reduced, but the total period of illness decreased from forty to twenty-five days. A similar serum was prepared by Kruse and its use reduced the mortality among Kruse's cases from 11 to 5 per cent. Kraus and Doerr also obtained favorable results from the use of their serum, which was mainly an antitoxic serum produced by the injection of filtrates of young cultures into horses. Vaillard and Dopter treated a large number of cases with a serum prepared by themselves and possessing both antibacterial and antitoxic properties and reported highly encouraging results with a mortality of 2 per cent., while the mortality otherwise would have been between 11 and 25 per cent. More prompt effects were obtained when the serum was given at the earliest moment in the course of the disease. Vaillard and Dopter used 20 to 30 c.c. in moderate cases and from 40 to 80 c.c. in grave cases. In late cases serum injections were often of value. Graham more recently has added a valuable contribution to the serum therapy in bacillary dysentery, his studies being made during the campaign in Macedonia. Graham used a serum prepared at the Lister Institute and gave intravenous injections of

60 to 80 c.c. once or twice daily during the first three days of treatment. Three injections were followed by 150 to 300 c.c. of saline daily for the first two days and once for the next two days, the saline injections being made to prevent dehydration of the tissues. In mild cases no saline injections were necessary. Most of the cases arrived after the third day, so that they were not placed under treatment at the earliest possible moment. On entering the hospital all cases received 20 c.c. of the serum subcutaneously. Klein also maintains that anti-dysenteric serum given early and in large doses intravenously (60 to 100 c.c.) is efficacious. He found that the use of the serum produced the best results when given during the first five or six days. When the disease has entered into the intermediate stage, from the sixth to the tenth day, the outcome of the disease is irrespective of serum treatment. In the third stage—tenth day—the use of serum is practically without value. Waller treated 140 cases with the Lister Institute serum and found that the early use of the serum resulted in shortening the duration of the disease. He gave three subcutaneous injections of 140 c.c. at eight-hour intervals during the first twenty-four hours to fairly severe cases. Rosenthal, in a series of serum-treated cases, found a mortality of 0.6 per cent. In other units the mortality was 10 to 15 per cent. Sixty c.c. of sera were given by Rosenthal on the first day, followed by 40 to 60 c.c. on the second, and if no improvement was observed subsequent doses of 40 c.c. were given up to a total of 400 c.c. Usually the stools were free of blood in forty-eight hours, and their number reduced from 60 to 15 or 10 per day. Lantin also thinks that the use of serum constitutes an efficient specific method of treatment. He gave the serum by rectum in doses from 30 to 50 c.c. Neumann used human convalescent serum in 400 cases. Intestinal irrigations with silver solutions were also employed by this author. Only six of his cases ended fatally. Jacob, on the other hand, and with him also Nolf, failed to obtain success with serum therapy in this disease. Jacob treated ninety cases, using polyvalent sera and injecting subcutaneously or intravenously doses ranging from 20 to 400 c.c. during the first or second week of the disease. According to the British Medical Research Committee, serum treatment of bacillary dysentery is not satisfactory. Nevertheless, numerous investigators showed that this method of treatment has a well-established clinical value as expressed in the view of Schittenhelm, who states that it should be employed in all cases of more than three or four days' duration, and in all cases showing toxemia and severe symptoms, and in cases where the number of stools are more than twelve in the course of twenty-four hours. It should be given early in the disease and in massive doses. If possible the type of the infecting organism should be known prior to the administration of these massive doses. This can readily be done in twenty-four hours in a well-equipped laboratory. The serum used should be polyvalent, because there are a number of

serologically distinct types of dysentery bacilli. Schmitz, for instance, found in a dysentery outbreak among prisoners of war in Roumania strains which resembled the Shiga bacillus but were serologically entirely distinct types. Pribram also found that an antitoxin specific for the Shiga-Kruse toxin is inactive toward the toxin of a strain D¹¹⁸H (Hallmann). Furthermore, the curative action of anti-dysentery serum is due first to its content in antitoxin, and second to its anti-bacterial properties.

The serum can further be employed for prophylactic injections in doses from 10 to 30 c.c., but the immunity thus produced will be only of a short duration. Recently Boehneke and Elkeles have inoculated over 100,000 persons with a polyvalent bacillary toxin-antitoxin preparation called dys-bakta, but complete protection was not secured. It was noted, however, that infections occurring in the inoculated individuals were usually of slight severity, and death a very unusual occurrence. The reaction following inoculations was no more severe than that following typhoid inoculations. Immunity thus produced lasted for at least three months.

Botulinus Toxin and Antitoxin.—*Bacillus botulinus* produces a toxic body leading to symptoms often called "ptomaine poisoning." The toxin, however, is apparently independent of the medium used, is destroyed by moist heat of 58° C. for three hours, and of 80° C. for one-half hour, and is capable of inducing the formation of an antitoxin. The symptoms produced by the toxin are marked increase or decrease of saliva flow, vomiting, sometimes diarrhea, but more often constipation, often retention of urine, paralysis of eye muscles, aphoria, rarely fever or disturbance of sensitivity. Death frequently ensues following the appearance of symptoms of bulbar paralysis with disturbances of respiration and heart action. The necropsy shows marked general passive congestion and thrombosis of the meningeal vessels sometimes with slight hemorrhage. Unlike other toxins, that of botulism resists the digestive juices and is absorbed by way of the alimentary canal. It can be neutralized by brain substance and by the lipoids, lecithin, cholesterol, and by fats, such as butter and oil. It is toxic for man, monkey, cat, rabbit, and guinea-pig.

The Use of Immune Sera in Botulism.—Van Ermengem in 1895 discovered the cause of botulism poisoning to be an exotoxin produced by a strictly anaërobic Gram-positive bacillus which he isolated from portions of a ham that had caused fifty cases of poisoning at Ellezelles, Belgium. The disease has an exceptionally high mortality of almost 100 per cent., and up to the present time the percentage of fatal cases has been as great as it was fifty years ago. The reason for this lies in the fact that the early symptoms of the disease are not recognized until the toxemia is well established. In the year 1897 Kempner showed that susceptible animals may be successfully immunized to the toxins of this organism and obtained a potent antitoxin from goats, 1 c.c. of the serum protecting against

100,000 minimal lethal doses. Forssman and Lundstrom were also successful in their immunization attempts, using attenuated toxins. Wassermann immunized horses and produced sera of undeniable value in animal experiments. In this country sera were prepared by Graham, Brueckner and Pontius, Buckley, Hart, Meyer, Hurwitz and Taussig, Burke, Dickson, and Howitt mainly for experimental purposes, using rabbits, sheep, goat, cattle, and dogs for immunization. According to Dickson and Howitt, laboratory experiments show that the antitoxin may protect against the action of the toxin for at least twenty-four hours after the administration of one test dose of toxin, but the effectiveness is, to a certain extent at least, dependent upon the amount of toxin injected. Like tetanus antitoxin, botulinus antitoxin should be given early if it is to be effective, and even in well-established cases it is strongly advisable to give antitoxin in massive doses, because Kob has demonstrated that this toxin may persist in the blood nine days after the poisoning. If symptoms of botulism, such as hypersecretion of mucus from mouth and nose, visual disturbances, aphonia, dysphagia, and paralysis of the intestinal tract appear, antitoxin should be administered as soon as possible, and should be given in large doses intravenously. Dickson also advises the use of antitoxin to all persons who have eaten fowl that have suffered from limberneck. Of importance is the use of polyvalent sera because of the discovery of Leuchs that two strains, the one of Van Ermengem and a Darmstadt strain, were distinct, that the toxin of one was not affected by the specific antitoxin of the other, and *vice versa*. As for the effect of botulinus antitoxin in man, little is known, as it has been used only in isolated instances. Dickson and Howitt, in 1918, gave 85 c.c. of immune goat serum (1 c.c. equivalent to 3000 M.L.D. for a guinea-pig) to each of two patients at Madera, California. Both patients recovered, but as the antitoxin was given very late, in fact, after all the more seriously poisoned patients had succumbed, there is no definite evidence that the course of the illness was favorably influenced by the antitoxin, although it was later shown that the toxin of the strain recovered from the food was Type A. McCaskey used small doses of antitoxin in three patients (5 to 10 c.c.). One died and two recovered and this author therefore thinks the serum to be of some aid. Nonnenbruch obtained rapid improvement in his case after the use of antitoxin. His patient became poisoned after eating sausage. Jennings, Haas and Jennings in the recent Detroit outbreak used Graham's serum in a dose of 42 c.c. intravenously in one case without apparent effect, and 20 c.c. in two injections to another patient, who recovered, and state that the latter case was not of mild type. Dickson and Howitt found that of all the outbreaks in which the serum had been used, with the exception of the cases of McCaskey, the toxin was that of Type A, and consequently when Type B serum was used it could not be expected to give any satisfactory results. As it is impos-

sible to determine quickly the type of toxin in a particular outbreak, it is of the greatest importance to use polyvalent sera.

Gas Bacillus Toxin.—The frequent occurrence of gas gangrene in the Great War has given especial interest to the preparation of antitoxins for the organisms causing the disease. Klose, in 1916, and Bull and Pritchett, in 1917, were able to prepare a soluble toxin of the bacillus *Welchii* or as it is often named bacillus *perfringens*. Bull and Pritchett drew especial attention to the necessity for selecting a strain which is capable of producing toxin in fairly large amounts. The British Medical Research Committee reports that the toxin of vibron septique has very little effect following subcutaneous injection. Upon intravenous injection, however, it produces convulsions and usually death in a few minutes. An antitoxin may be produced, but it is not effective after the toxin has been injected. The toxin of bacillus *edematiens* produces massive edema about the site of inoculation. The toxin of bacillus *aerogenes capsulatus* was found to have a necrotic action upon the tissues; it is generally toxic in large doses and animals may be protected by antitoxic serum.

The Use of Immune Sera in Gas Gangrene—Treatment of the Disease.—Leclainche and Vallée, Sacquépee, Weinberg and Séguin, Bull and Pritchett were the first to apply serum therapy in wounds infected with the gas bacilli. Leclainche and Vallée's and Weinberg and Séguin's serums were polyvalent and also antibacterial, while Bull and Pritchett's serum was antitoxic. In 1917 Bull and Pritchett produced an exotoxin from twenty-four-hour cultures of bacillus *aerogenes capsulatus*, which when injected into pigeons or guinea-pigs caused local edema, necrosis, and hemolysis of red cells, and was capable of stimulating the formation of an antitoxin. Bull's claim for the potency of his antitoxic serum was based on experiments in which he used pure cultures of bacillus *aerogenes capsulatus* and made no attempt to discriminate between the different types of the organism, such as have been found to exist by Henry, or to consider the fact that in war wounds the bacillus *aerogenes capsulatus* is not the only causal factor of gas gangrene. From Nevin's work it would appear that neither anti-*perfringens* serum (bacillus *aerogenes capsulatus* anti-microbial serum) nor Bull's antitoxin afford any protection when other pathogenic anaërobes incident to war wounds are present, together with bacillus *aerogenes capsulatus*, whereas when the vibron septique and bacillus *edematiens* are present in mixed infections without bacillus *aerogenes capsulatus*, the prophylactic use of the specific sera, even when diluted by another serum, is effective. Weinberg and Séguin, who have contributed extensively to the serum therapy of gas gangrene, found treatment by serum alone limited because of rapid absorption of toxin in this disease. The association of rational surgery and of serum therapy gives the best results. In a series of sixty-six cases reported by these authors in which sixty did not

receive serum treatment, three received non-specific treatment and three suffered complications, thirty-five deaths were recorded, while in a series of twenty-four specifically treated cases only five deaths occurred, thus reducing the mortality from more than 55 per cent. to less than 21 per cent. The serum used in these cases was polyvalent, produced against bacillus aerogenes capsulatus, the vibron septique, and bacillus edematiens. Duval and Vaucher, in 1917, reported fifty cases in which a combination anti-perfringens, anti-edematiens, and anti-vibron septique serum prepared by Weinberg and Séguin was injected prophylactically. In none of these patients did gas gangrene develop, although all were of the most severely wounded type. Twenty-five died as a result of severe multiple wounds without any signs or symptoms of gas gangrene.

Prophylactic Use of Sera.—A year later these same authors reported a series of 281 cases in which severely wounded patients were injected with polyvalent serum prepared at the Pasteur Institute. Eighteen developed gas gangrene (6.4 per cent.), and of these ten died, resulting in a mortality of 3.5 per cent., the usual mortality from gas gangrene in severely wounded being 16 per cent. Mairesse and Regnier found among 1016 wounded men examined bacteriologically 297 gas bacillus infections. They received prophylactic injections of anti-serum depending on the type of organism present. In thirty instances, or 10 per cent. of the cases, the disease developed. Ivens also used Weinberg and Séguin's serum in 222 cases for prophylactic injections. Among these no deaths occurred, and fourteen amputations were performed without fatal results. With Leclainche and Vallée's serum (154 cases) four died, and in fifty-seven other cases treated with both sera two deaths occurred. Further favorable reports were made by Quénu, Bazy and Routier, Vincent and Stodel, Marquis, Dufour and Samelaigne. Curative injections were given by Duval and Vaucher with 20.7 mortality. Rouvillois, Guillaume, Louis, Pedeprade, and Thibiérge treated twenty-five cases, five of whom died. Of these three were moribund on entrance to the hospital. Mairesse and Regnier's thirty treated cases had a mortality of 16.6 per cent.

Van Beuren, who reports a personal communication from Lieut.-Col. W. Elser, states that prophylactic doses were given to 15,000 soldiers and controlled by 15,000 others. According to this finding there was not sufficient difference in the incidence rate to warrant any definite declaration as to the protective value of the sera used. Apparently these investigators were favorably impressed; for they laid the failure to secure better results to the weakness of the serum then available. Elser advises the following routine for the serum treatment:

1. A prophylactic dose of polyvalent serum, combined with tetanus antitoxin, given as early as possible after the receipt of the wound.
2. Bacteriologic examination of the wound and establishment of

the presence of gas bacillus infection and determination of the variety of the bacteria.

3. Administration of specific serum, either single or polyvalent or "pooled," according as there are one or more gas formers found, and also the administration of anti-streptococcus serum, since the latter organism is very commonly found in association with the other organisms.

From the general reports obtained during the Great War it is considered that intravenous injection is to be preferred, in combination with deep muscular injections in the vicinity of the wound. From these reports it seems, then, that the use of a polyvalent anti-bacterial and antitoxic serum is advisable, but much work on the subject must yet be done. From all the observations at hand it is safe to state that the best results are to be obtained from preventive injections.

Bacterial Hemotoxins.—As an example of the hemotoxins produced by bacteria certain details of staphylolysin may be considered. The hemotoxin is produced by twelve to thirteen days' growth of *staphylococcus pyogenes aureus* or *albus* in broth. The organisms are killed and the broth filtered through a porcelain filter. The filtrate can be preserved by the addition of 5 per cent. of a solution made up of 10 parts phenol, 20 parts glycerol, and 70 parts water. Doses of 0.025 to 0.05 c.c. should completely hemolyze one drop of rabbit blood after two hours at 37° C. Antilysin may be produced by immunization of animals and is found normally to a slight extent in normal human blood and in that of certain lower animals. The victims of *staphylococcus* infections frequently show an increased antilysin content of the serum. This fact has been employed by Bruck, Michaelis, and Schultze to diagnose *staphylococcus* infections, some cases showing increases of ten to one hundred times over the normal antilysin. The simplicity of bacteriological examination, however, makes this method of diagnosis by comparison rather cumbersome and time consuming. Whether or not antilytic sera would be of value in the treatment of those cases that resist or are unsuitable for vaccine treatment has not been determined so far as we have been able to learn.

PHYTOTOXINS

Introduction.—Although literally the phytotoxins include all the toxins of vegetable origin the term usually is restricted to include those originating in forms of vegetable life higher than the bacteria. With this definition thought would be first directed to the poisonous fungi, but as has already been shown, only one of the poisons so far isolated is capable of inducing antibody formation. The poisonous elements of poison ivy and poison oak produce no antibodies. The poisonous elements of those plants that produce "hay fever" require separate discussion, because the toxic factor operates only on individuals who show a peculiar susceptibility or "hypersus-

ceptibility." The element of hypersusceptibility in this connection will be deferred until after the presentation of the fundamental material on anaphylaxis and hypersusceptibility. The following paragraphs will present briefly the essentials concerning ricin, abrin, robin, crotin, curcin, and phasin. This brevity is justified by the relatively small practical importance of these substances.

Ricin is the toxic principle of the castor-oil bean, *ricinus communis*. It was isolated by Gibson in 1887 and named ricin by Stillmark in 1888. Cushing made very strong toxic preparations and Field states that ricin will kill rabbits in doses of 0.0001 mg. per kilo; guinea-pigs, 0.0008 mg.; dogs, 0.0006 mg.; cats, 0.0002 mg.; and goats, 0.003 mg. Following injection there is an incubation period succeeded by diarrhea, somnolence, weakness of extremities, and death. At the necropsy are found reddening and swelling of Peyer's patches, mesenteric and retinal hemorrhages, ulcers of stomach, nephritis, general lymphatic swelling, and softening and degeneration of the pyramidal cells of the cerebral cortex. Beauvisage reported 150 cases of ricin poisoning in man of which nine were fatal. Many of these were children who ate the seeds, but there were also soap makers who handled the beans in soap manufactories. Ricin and the other toxins in the group may be precipitated with the proteins by ammonium sulphate; they are precipitated by alcohol and are gradually destroyed by proteolytic enzymes. Jacoby, however, claims to have produced ricin and abrin which failed to give protein reactions. Osborne, Mendel, and Harris maintain that ricin is inseparably associated with protein, and that Jacoby's error was due in all probability to the fact that he obtained a product so toxic that the small amounts necessary for toxic action were too small to give the protein reactions. The most striking character of ricin *in vitro* is its capacity to agglutinate the red blood-corpuscles of practically all warm-blooded animals. It may agglutinate other body cells, precipitates protein, and is adsorbed by casein, fibrin, coagulated serum albumin, and by silk. Jacoby concludes that ricin is a mixture of agglutinin and toxin, the two having certain molecular groups in common. Ehrlich believes that these may undergo alteration into agglutinoid and toxoid. The mechanism of the agglutination is not clear and many hypotheses, none quite satisfactory, have been advanced. Ehrlich produced an antiricin by giving increasing doses to animals by mouth, and then changing to subcutaneous injections. This antiricin was used by Ehrlich in the development of much of his hypothesis of the toxin antitoxin union because of the ease of manipulation as compared with the time-consuming and expensive method of working with animal injections of toxin antitoxin mixtures. In addition to the antitoxin there are present in the serum a closely related antiagglutinin (with which Ehrlich worked) and a precipitin for ricin solutions.

Abrin is obtained from paternoster or jequirity bean, *abrus precatorius*, and was described by Warden and Waddell in 1884. It is

much less toxic than ricin, producing gastro-enteritis, hemorrhages, and swelling of lymph-nodes. Local applications led to an acute conjunctivitis and in hairy regions to transitory loss of hair, both of which may be protected against by immunization. Kobert states that in India and Ceylon cattle were immunized (by feeding beans) against the effects of wounds by abrin-coated projectiles. Roemer found that by repeated application to the conjunctival sac of one eye, he could produce an immunity which first protected that eye and, after further immunization, served to protect the opposite untreated eye, in this later stage becoming a general immunity with antiabrin in the serum. Abrin is also a hemagglutinating agent, and can be distinguished from ricin by immunological experiment. In many respects abrin and its immunity resemble ricin very closely.

Croton is derived from croton seed, croton tiglium, and is less toxic than either ricin or abrin. According to Elfstrand, it agglutinates the red blood-corpuscles of beef, sheep, swine, and frog; it hemolyzes the cells of rabbit, cat, and crow, and has no effect on the erythrocytes of man, dog, guinea-pig, rat, hen, goose, and pigeon. Immune sera can be produced by the usual methods. Jacoby found in Grubler's pepsin a body which he called pseudo-anticroton, capable of neutralizing the action of croton on erythrocytes *in vitro* but not *in vivo*, and he found the same substance in gastric and intestinal mucosa.

Curcin is produced from the seeds of *jatropha curcus*, and *robin* from the leaves and bark of *robinia pseudacacia*. Immune sera can be produced against both of these.

Phasin is a name given by Landsteiner and Raubitschek to a hemagglutinating substance found in the seeds of the bean, pea, lentil, and vetch. Antiagglutinins are found in normal serum and may be increased experimentally, but this substance or group of substances can hardly be regarded as belonging to the class of toxins because of little or no toxic symptoms following injection.

Pollen Proteins or Pollen Toxin.—The modern studies of hay fever and of asthma place this subject so clearly in the group of anaphylactic phenomena that its consideration is deferred (see page 233).

ZOÖTOXINS

Introduction.—The zoötoxins include the poisonous elements produced in animal life. They may be, and most frequently are, in the form of excretions of special poison glands or are found in secretions of other glands, in blood and in tissues. The most important are the snake poisons, but there are also included the poisons of spiders, scorpions, bees, centipedes, tarantula, toads, poisonous fish, duck-bill platypus, and the sera of various animals.

The snake venoms differ somewhat in their action according to family, the colubridæ, including the cobra, Australian black snake, and others; the viperidæ, including the European viper and Ameri-

can rattlesnake; and the hydrophinæ or poisonous sea snakes. The venoms secreted by special glands are injected during the bite through fine canals in the fangs (not the forked tongue), and are all hemolytic. The fact that the blood of snakes contains poisons similar to those of the venom indicates that the poison glands secrete with little alteration the poison of the blood. Nevertheless, snake bites may be poisonous for snakes of other species, and also for other members of the same species. Geoffroi and Hunauld, in 1737, and Fontana, in 1781, noted the anticoagulant action of venom, but the work of Weir Mitchell in 1860, and of Weir Mitchell and E. T. Reichert in 1886, served as the greatest stimulus to modern investigation. Mitchell and Reichert showed that the venom of the rattlesnake produces rapid coagulation of the blood and death, but that if the animal survives the blood is reduced in coagulability. C. J. Martin confirmed this in regard to Australian snakes and showed that the phenomenon could be controlled by dosage of the venom. In addition to hemolysis and alteration of coagulation, other properties are present, and Flexner and Noguchi showed in venom the presence of hemotoxins, including hemolysins and hemagglutinins, leucocytolysins, and an endotheliotoxin which they named hemorrhagin. Pearce showed that hemorrhagin produced lysis of endothelium leading to hemorrhage. In addition, venoms contain proteolytic enzymes, invertase, lipase, and probably certain ferments dealing with coagulation. Martin found fibrin ferments which probably aid in thrombus formation. Lamb found that even citrated blood could be clotted by venoms. Negrete found the anti-coagulating element closely associated with the proteins of the venom. Morowitz claims the presence of an antithrombokinase. Modern studies by Houssay Sordelli and Negrete with the venoms of fourteen snakes, Indian, American, and Australian, show that clotting time does not parallel closely the dose of venom, that venoms clot whole blood, plasma, and fibrinogen solutions, and that mammalian blood is more susceptible than that of birds, batrachians, and snakes. The addition of citrate, oxalate, magnesium sulphate, hirudin, and peptone delay the action of the venom, the oxalate acting the most intensely. It seems likely that large doses of venom bring to bear a sufficient amount of fibrin ferment to produce clotting and that the later effects are due to the anti-coagulating power of the venom after the fibrin ferment is exhausted. Inasmuch as the hemolysis of venom is somewhat closely related in mechanism to hemolysis in general, it will be discussed in the chapter on Hemolysis (see page 141). Venom toxins resemble other toxins in that they are precipitated with proteoses, whilst the factor which produces local irritation comes down with globulin, although Faust maintains that the active principles of venoms are glucosides. Venom toxins are destroyed by heat, the cobra poisons as a class by 100° C., and the viper poisons by 85° C. They do not dialyze and deteriorate under the

influence of light, radium, and oxidizing agents. There is an incubation period and the venoms are definitely and specifically antigenic.

Venoms act in extremely small amounts. The fatal dose of cobra venom for man is probably 0.01 to 0.03 gm., rattlesnake venom 0.15 to 0.3 gm., and poisonous sea snakes 0.001 to 0.003 gm., or ten times as toxic as cobra venom. The bite of the cobras produces little pain and local reaction, probably due to its small content (2 per cent.) of globulin, which contains the local irritant property of the venom. A feeling of stiffness spreads from the region of the wound, followed by vertigo and weakness of muscles of locomotion, tongue, jaw, esophagus, and preservation of senses, resembling a very acute bulbar palsy with death in a few hours. Cushing, however, finds that the action of the poison is particularly upon motor nerve termini. The venom of the vipers produces a marked local reaction, probably due to its large (25 per cent.) globulin content, with pain, swelling, local bleeding, blood in the serous membranes and hematuria. Nausea and vomiting, excited reflexes, and even convulsions are followed by prostration, paraplegia of lower extremities which extends upward and resembles an acute ascending spinal paralysis with death in one to three days. Langmann states that, "if the patient recovers from the paralysis, a septic fever may develop; not rarely there remain suppurating gangrenous wounds which heal poorly." The suppuration of snake bites (vipéridæ) has been the subject of considerable study; Welch and Ewing ascribed this to loss of bactericidal property of the blood after venom poisoning. Flexner and Noguchi demonstrated a loss of the complement of the blood, an element necessary to its full bactericidal power. They believed that the complement was used up by the venom whose amboceptors require complement for their action, therefore leaving little or none free for the bactericidal amboceptors. Morgenroth and Kaya claim that the complement is actually destroyed by the venom. Of considerable importance in favoring infections must be the local necrosis of tissue caused by the venom and the associated hemorrhage, aided by the customary radical surgery of the wound.

The production of antisera was placed on a practical basis by Calmette in 1894 and Frazer in 1895. Calmette attenuated cobra venom for the first four injections by the addition of equal volumes of 1 per cent. gold chloride solution, and then gave small doses of the native toxin, gradually increasing until a powerful antivenin was developed. Phisalix and Bertrand attenuate viper venom by heating the first dose to 75° C. and then after two days giving one-half the minimum lethal dose of toxin. It was at first thought that the antivenin produced by cobra venom would protect against all venoms, but it was soon shown that the sera were specific for the venom employed. Antivenin also neutralizes that element of venom which induces loss of bactericidal power of the blood. Noguchi has shown that the antivenin of rattlesnake venom neutralizes the

hemorrhagin. Such sera also contain precipitins for the proteins of the special venoms employed and for the serum proteins of the same species of snake. These are highly but not absolutely specific. The mechanism of venom-antivenin union is probably very closely similar to that of toxin-antitoxin unions of other varieties, although Kyes holds that the former is distinctly in the nature of the chemical reaction between a strong acid and a strong base.

Scorpion poison is secreted by special glands in the abdomen. In human adults the symptoms are rarely severe, except for marked local reaction, but it is stated that the bite of an African scorpion may kill children. As a rule, the most serious effects are from the subsequent infection of the wounds. Todd was able to prepare a specific immune serum for the poison of scorpions. According to Houssay, scorpion venom acts pharmacologically much as veratrin; it is a smooth muscle stimulant. He states that serum therapy is useful and specific.

Spider poison is secreted by glands in the thorax. The common spiders are not venomous, except the "cross spider" whose venom, much weaker in the saliva than in the ovaries, closely resembles snake venoms in chemical properties and agglutinin, and probably contains a neurotoxin. Sachs prepared an antivenin against this venom. Some of the larger spiders are extremely poisonous, particularly the Malmigatte of southern Russia and related species in South America and Africa. Large numbers of cattle have been poisoned with as high as 12 per cent. mortality, but the bite is rarely fatal for man.

The tarantula produces a poison which operates almost entirely locally, and it is stated that an antitoxin can be produced against the Russian tarantula.

Centipedes.—Certain centipedes secrete a poison in special glands that discharge through the claws, capable of producing considerable local reaction. But one case of fatal poisoning has been reported from Texas, that of a child four years old.

Bees, wasps, and hornets secrete a poison closely similar for all three. Bee poison contains formic acid and in addition a poison which does not give the usual protein reactions, but is destroyed by proteolytic enzymes; it resists heat to 100° C., weak acids, and alkalis. The poison contains a hemolysin which operates in much the same manner as does cobra hemolysin. The bite produces marked local reaction, but only in cases of extreme hypersusceptibility are there general effects or death. Part of the lack of severity of bee poison is due to the small dose injected, for if collected in large amounts and injected intravenously into dogs it can produce death. The resistance of professional bee keepers to the bites is probably due to the fact that repeated bites lead to development of immunity, although it is possible that the doctrine of the survival of the fittest may play its part. Ants probably pro-

duce a somewhat similar poison in addition to formic acid. The "black flies" of the woods produce a poison not as yet identified, but no poison has as yet been isolated from the mosquito. Numerous other insects appear to have poisonous secretions, but as yet no studies have been made in detail as to their isolation and identification.

Toads, frogs, and salamanders produce dermal secretions which are poisonous, several of which operate like digitalis and some like epinephrin. These poisons are interesting from a pharmacological point of view, but as they are not capable of producing immune reactions in animals they deserve no extensive discussion here.

Poisonous fish comprise several groups. One group secrete poisons in special glands, the poison being discharged through spines. Such poisons contain a hemolysin which requires an activator, as in the case of cobra-venom hemolysins. These poisons act as powerful local irritants and as cardiac depressants and may cause death. Only one variety of fish produces poisoning by its bite, the poison being secreted in the gums. Other fish are poisonous when eaten even when quite fresh, the poison being found especially in the ova and ovaries. The symptoms may be of a severe choleriform type frequently fatal, or of a less severe gastrointestinal type, not commonly leading to death. Certain fish, particularly in the tropics, rapidly decompose with the formation of poisonous products or ptomaines. The bites of crabs may produce peculiar erysipelas-like lesions or "erysipeloid," but the origin and nature of the poison are not known. Many individuals develop toxic symptoms after eating shell-fish and other sea food, in some cases due to the decomposition of the food, but in most instances due to a peculiar hypersusceptibility which will be discussed under Hypersusceptibility (see page 230).

Eel serum deserves special consideration because of the fact that immune bodies can be produced. It is not poisonous when ingested, but is highly so if given intravenously and it produces conjunctivitis when instilled into the sac. Relatively large doses lead to rapid death and small doses may produce cachexia and death after several days. The toxic element is in the albumin fraction of the serum and is destroyed by 58° C. for fifteen minutes. It contains a hemolysin and probably also a neurotoxin. The hemolysin does not act as an amboceptor, reactivation by fresh serum being impossible after the eel serum has been heated. Immune sera can be produced which neutralize the hemolysin *in vitro* and also protect animals from death by the eel serum. The serum of lampreys and rays is similarly toxic.

The parasitic protozoa and other animal parasites are strikingly free from substances which induce immunity. The protozoa show few exceptions to this rule. Cytolysins can be produced experimentally for amebæ, but no such reaction takes place in human patients. Active immunity to trypanosome infection can be pro-

duced, and it is claimed that immunity can be conferred passively. The trypanosomes, however, can become immune to trypanocides. Malarial parasites produce among other things a hemolysin, but there is no indisputable evidence that immunity occurs in malaria, nor have immune reactions been developed. Sarcosporidia of sheep produce a toxin fatal for rabbits in doses of 0.0001 gm., against which an antitoxin may be produced in rabbits. Complement-fixation reaction is positive in infested sheep. Man may be infested by the cyst of one tape worm, the tenia echinococcus, the cyst contents being definitely toxic, as shown when a cyst ruptures into a body cavity, *e.g.*, the peritoneum. Serum of infested patients contains a precipitin for the cyst proteins and also a complement-fixing body, Zapelloni reporting 93 per cent. positive complement-fixations in 500 cases examined. Of the adult tape worms which infest man the *dibothriocephalus latus* is the most important from the immunological standpoint, although this parasite is rare in America. The proglottids contain a thermostabile hemolytic lipid liberated on the death of the segments by auto-digestion. There is also a thermolabile hemagglutinin. It is probable that the hemolysin is either associated with other cytolytins or that a species cytolytin is present which also acts as a hemolysin. This is responsible for the primary type of anemia seen in *dibothriocephalus latus* patients. The serum of these patients contains a precipitin for the fluid obtained by antolytic digestion of the segments. Of the nematodes the ascaris, the trichinella spiralis, the hook worm, and certain forms of filaria have been investigated. Certain ascarids produce poisonous substances without immunological relations. In regard to trichinosis Salzer has found that the serum of recovered patients has distinct therapeutic value in infested patients and protects animals against experimental infestation. Complement-fixation has been found to be of value in the diagnosis of trichinosis. It has been claimed that the anemia of hook-worm infestation is due to a hemolytic poison, but there is doubt that this is as important as the small repeated hemorrhages produced by the bite of these parasites. There is little of immunological significance in the studies of the filariæ. The guinea-worm (*filaria medinensis*) contains in its body a violent irritant which may be discharged by rupture of the worm during forcible attempts at its removal, and leads to severe local inflammation and even to gangrene.

Mammalia do not produce poisons except in the somewhat questionable case of the male duck-bill platypus of Australia, a survivor of the very earliest forms of mammalian life. Special glands are said to secrete a poison like that of Australian snakes, which is discharged through a hollow movable spur on the hind foot. There is serious question as to the toxic properties of this secretion, certain authorities believing that the sequences of such wounds are due to infection and that the secretion is of importance only as a secondary sex character. The serum of certain mammals is toxic on injection,

as, for example, beef serum, which in doses of 0.5 c.c. will kill a guinea-pig in a few minutes. Dog serum is also toxic for guinea-pigs in somewhat larger doses (1.0 to 2.0 c.c.). Horse serum is toxic for cats in doses of 1.0 c.c. per kilo, and for guinea-pigs in doses of 20.0 c.c. per kilo., but man is practically insusceptible, except in those cases of hypersusceptibility in which small doses of serum produce serious symptoms and even death. Such toxic sera contain hemolysins and agglutinins in small amounts and reduce the coagulability of the blood, but death is probably due to other factors. Except for cases of natural or artificial hypersusceptibility, the toxic element is destroyed by heat of 56° C. and is removed by animal charcoal.

CHAPTER V

AGGLUTININS AND PRECIPITINS

GENERAL INTRODUCTION.

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BACTERIAL AGGLUTINATION.

INTRODUCTION.

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THE FORENSIC BLOOD TEST.

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ORGAN SPECIFICITY.

DETECTION OF FOOD ADULTERATION.

FUNCTION IN IMMUNITY.

General Introduction.—If a clear albuminous urine be boiled the invisible protein aggregates clump together, become visible as flocculi, and sink to the bottom of the test-tube. If to a colloidal suspension of mastic be added a proper concentration of common salt a similar flocculation of the mastic occurs. Red blood-corpuscles or bacteria shaken in physiologic salt solution form a cloudy suspension of particles invisible to the naked eye. They may be clumped together by a variety of methods in similar flocculi which become

clearly visible as small particles and sink to the bottom of the tube more quickly than would the individual cells in the original suspension. The first example is one of precipitation and the last of agglutination. In the immunological sense, precipitation implies flocculation of a protein solution by means of specific antibodies, so that large aggregates are formed and thrown out of solution. Similarly the term agglutination signifies clumping together by means of specific antisera of cells originally in smooth emulsion, so that the clumps are visible microscopically or grossly, and sink rapidly to the bottom of the containing vessel. Animals may be immunized to a protein in solution, as, for example, blood serum or egg white, so that the animal's serum contains a body, the precipitin, capable of precipitating the protein used for immunization. Similarly bacteria, red blood-corpuscles, or even other cells may be injected re-

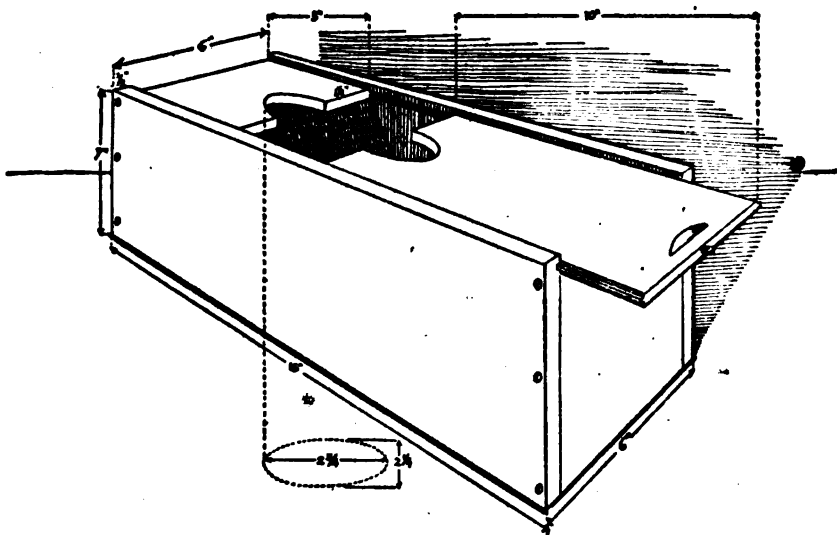


FIG. 3.—Wooden box for holding rabbits during injections into or bleeding from the ear vein.

peatedly into animals leading to the formation within the animal of a body, the agglutinin, appearing in the blood serum and capable of clumping the type of cell injected. These phenomena, although closely related and probably fundamentally identical in nature, will, for eminently practical reasons, be discussed separately.

AGGLUTININS

Bacterial Agglutination.—Although others had observed the phenomenon of agglutination, Gruber and Durham, in 1896, were the first to study it intensively in the course of work on the colon bacillus and the cholera vibrio. They pointed out the specificity of the reaction and the fact that it differed in certain essentials from previously studied serum reactions. These points will be discussed

in some detail, but it must be pointed out at once that the specificity is not absolute. It was soon found that blood-cells and later other body cells could be agglutinated by specific sera. It was also found that agglutinins of various kinds exist normally in certain sera, these being called normal agglutinins as opposed to the artificially produced or immune agglutinins. It was found that the agglutinins resist heat of 56°C. , a degree sufficient to destroy complement, and that after being rendered inactive by heat cannot be reactivated by fresh normal serum. It was soon observed that in the course of infectious disease due to a specific organism agglutinins are likely to develop, and this led to the discovery in Widal's

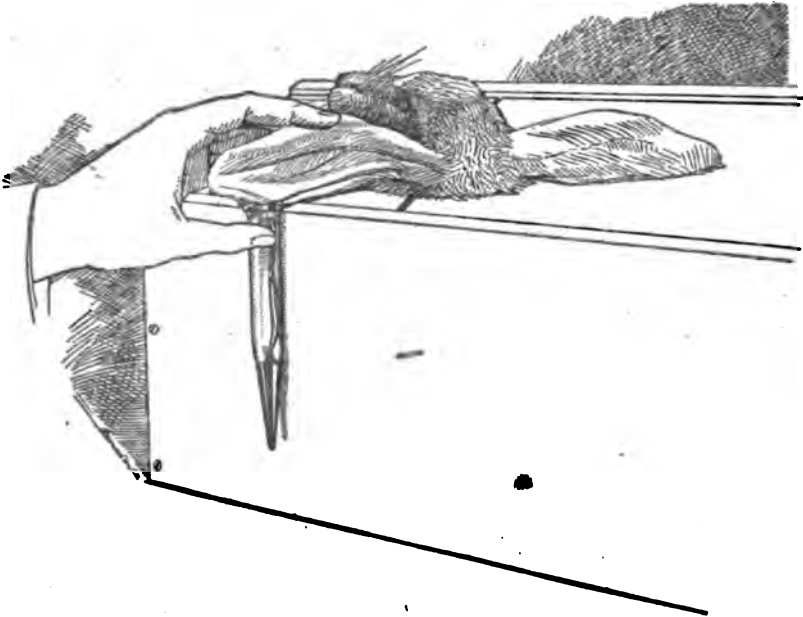


FIG. 4.—Method of obtaining blood from the posterior auricular vein of the rabbit's ear. The vein has been incised by means of a small hypodermic needle. The same position of the animal serves for intravenous injections which are given into the posterior auricular vein.

clinic in Paris, a few months after Gruber and Durham's publication, of the now widely used Widal reaction for typhoid fever. Conversely with a serum of known type, the antigenic bacteria may be identified. The demonstration of agglutination may be by the microscopic method or by the macroscopic method. In our presentation of the subject it is considered desirable to illustrate the points by actual experiment, and for this reason we proceed to take up the method of producing immune agglutinins in the laboratory and subsequently present the factors which qualify and modify the process of agglutination.

Production of Immune Agglutinins.—Injections for producing agglutinins may be subcutaneous, intraperitoneal, intravenous, or a

combination of these, using first the subcutaneous or intraperitoneal routes followed later by intravenous injections. Bacteria are usually killed by heat or chemicals before injection, although after immunization is well under way living organisms may be employed. The use of living organisms is often of service in the development of a serum of high titer.

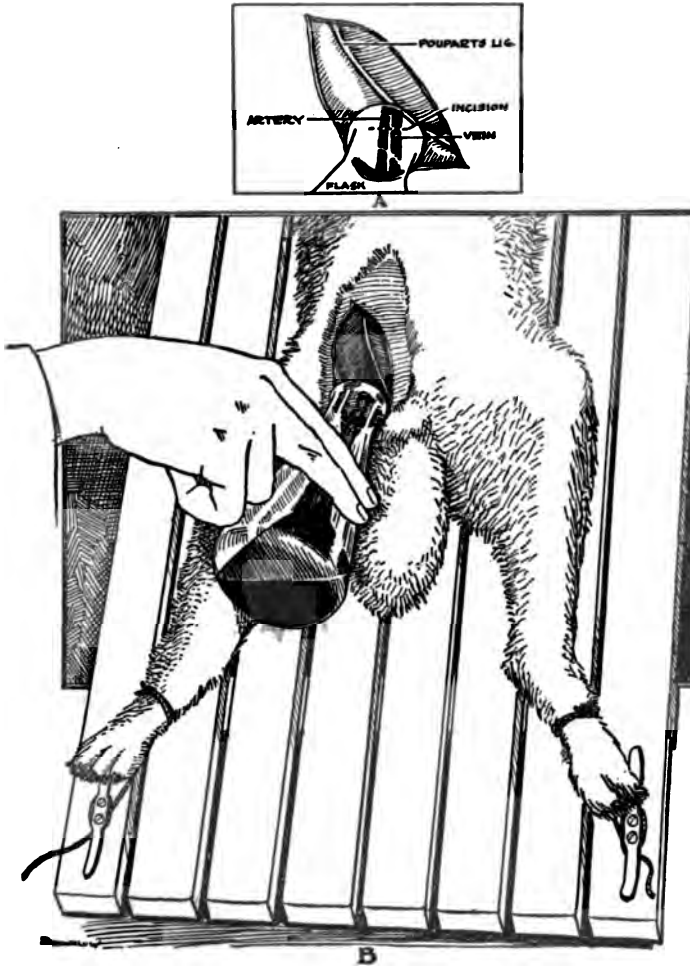


FIG. 5.—Method of complete bleeding from the femoral vessels of the rabbit (see text page 83).

The following will serve as a fairly typical example of the process of immunization for the production of an anti-typhoid agglutinin. The cultures used are twenty-four hour agar slants inoculated by zig-zagging the loop back and forth over the surface so as to have the surface well covered by growth. A measured amount, 5.0 c.c. or 10.0 c.c. of sterile salt solution is added, the tube allowed to stand ten or fifteen minutes and then vigorously rotated between the palms of the hands. This procedure gives a much smoother emulsion than washing off by sucking in and blowing out from a pipette or by scraping off with a platinum loop and is less susceptible to

contamination. The suspension is pipetted into a sterile tube and the growth killed by placing in a water bath of not less than 56° C. or more than 60° C. for two hours. Rabbits are desirable animals because of the ease of intravenous injection. For ease in handling, the animal is placed in a box as shown in Figs. 3 and 4. The ear is shaved along the course of the posterior auricular vein situated near the posterior margin of the ear on its upper surface, is cleansed with soap and water and sponged over with alcohol. Usually the alcohol makes the vein stand out prominently, but if it does not, the ear may be pinched near its root so as to distend the vein, or, if necessary, brushed over lightly with a sponge dipped in xylol. Xylol should be used very sparingly, because of the danger of an inflammation, which may make subsequent injections difficult. Bleeding from the puncture may be stopped by pinching the ear for a few moments at the site of injection. Usually one ear is used for injections and the other for



FIG. 6.—A flask placed upright after blood has clotted with oblique surface. The serum drains to the bottom of the flask and is easily withdrawn.

test bleeding. The earlier injections or bleedings are near the tip of the ear, the later ones approaching the base. The following protocol illustrates an immunization:

Day	Killed typhoid emulsion
1	0.05 agar slant *
6	0.1 agar slant
11	0.2 agar slant
16	0.2 agar slant
21	0.2 agar slant

* If 10 c.c. saline had been added to the culture, 0.5 c.c. suspension would contain 0.05 agar slant.

Preliminary Titration.—One week after the last injection 0.5–1.0 c.c. blood is withdrawn from an ear vein and the serum separated and titrated for the agglutinin. (See Fig. 4.) If the titer is not satisfactory, the immunization may be continued with living bacilli as follows:

Day	Living typhoid emulsion
1	0.05 agar slant
4	0.1 agar slant
8	0.2 agar slant

After 7-10 days a further titration is made, and if still unsatisfactory the animal is discarded. As a rule, three animals are employed, and from these at least one will produce an agglutinin which will titrate 1-5000 or higher. The titer may be maintained by subsequent injections at longer intervals, but it is usually found desirable to kill the animal by bleeding and to preserve the serum in ampoules in the refrigerator.

Bleeding the Immune Rabbit.—The rabbit may be "bled out" by strapping it on a flat board, lightly anesthetizing and plucking the hair from the groin on one side. The skin is scrubbed with soap and water and then alcohol, and a long incision made in the line of the groin groove.

(See Fig. 5.) This goes through the fascias and exposes the femoral vessels. The neck of a sterile 150 c.c. flask is placed over the vessels just below Poupart's ligament and the vessels cut with the knife, the blood being caught as it spurts. As the bleeding continues the head end of the board is raised and the animal's body squeezed until the flow ceases. Before disposing of the body, death should be assured by a blow fracturing the cervical spine. The flask is placed in an oblique position until the blood is firmly clotted, then placed upright in the refrigerator. (See Fig. 6.) This leaves an oblique surface of clot from which the serum flows out in the bottom of the flask. The blood may also be obtained from the carotid artery, but this requires more careful dissection, longer anesthesia and may require the insertion of a cannula. Practically it gives no better results either in quantity of blood withdrawn or sterility of the process. Usually 15-20 c.c. serum are obtained after twenty-four hours in the ice-chest, and only occasionally is it necessary to centrifuge in order to obtain a clear serum. The serum is withdrawn as shown in Fig. 7 and placed in small sterile ampoules of dark glass, sealed and kept in the refrigerator.

Macroscopic Titration.—Titration is usually by the macroscopic method, but an alternative is the microscopic method. For the titration by the macroscopic method it is necessary to have the growth from two or three agar slants, adding about 10.0 c.c. sterile salt solution to each tube and making an emulsion as described for immunization. The suspension is placed in a flask and killed either by heat (56° C.-60° C. for two hours), or by phenol 1.0 per cent. or formalin (40 per cent.) 1.0 per cent. The killing of the organisms is not necessary, but is desirable because of the added safety and because such killed emulsions may be preserved in the refrigerator for several days or a few weeks. Broth cultures may be used, but the hydrogen ion concentration of the broth may add a small factor of error not present in the saline suspensions.

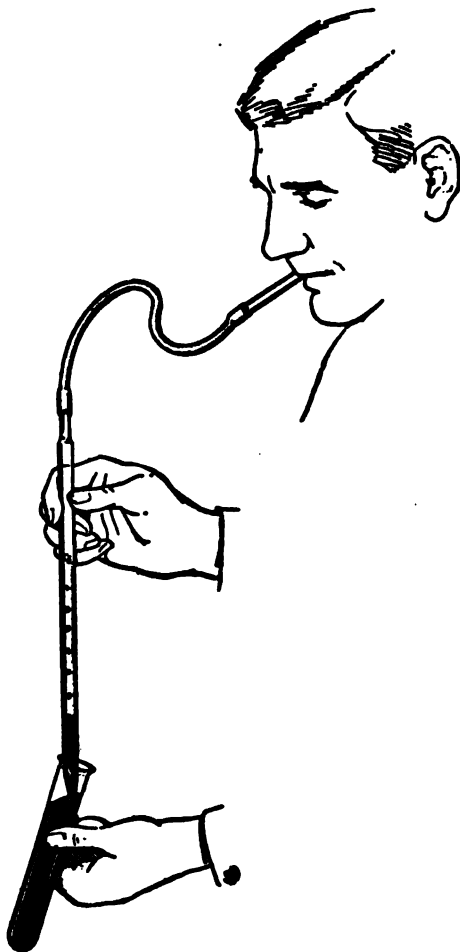


FIG. 7.—Method of drawing up measured volumes into a graduated pipette. The rubber tube enables the worker to observe the ascent of fluid in the pipette and the position of the tip of the pipette. Fluid is withdrawn from flasks in the same fashion.

Dreyer, who has given much attention to agglutination in the diagnosis of typhoid and paratyphoid fevers in individuals who have been vaccinated against these diseases, maintains that heat and chemicals other than formaldehyde are inferior to the latter in killing and preserving the bacterial suspension. He has given great attention to standardization of the reaction, an important but not infallible precaution, where a patient, as in the army, is likely to be examined in different laboratories during the course of the disease. On this basis Dreyer has shown that saline emulsions from agar cultures are inferior to broth cultures. Scheimann maintains in addition that the broth cultures furnish a more permanent standard. Laboratories in which such standards are prepared determine the optimum density of the agglutinable cultures and also keep the emulsions until the early deterioration of agglutinability produced by the formalin has reached a stationary point, after which the standards remain practically unchanged for ten months and probably longer.

The primary test is carried out in small test tubes, with each dilution one-half that of the preceding one. This simplifies making the dilutions, especially if only one serum is to be tested. A row of twelve tubes is placed in a rack and each tube receives 0.5 c.c. salt solution. To the first is added 0.5 c.c. immune serum, the mixture blown in and out of the pipette three times and 0.5 c.c. transferred to the next tube, the processes repeated and 0.5 c.c. transferred to the next tube, and so until the last tube is reached. In order to preserve the constant volume in each tube, 0.5 c.c. is discarded from the last tube. Thus there are dilutions 1-2, 1-4, 1-16, 1-32, 1-64, 1-128, 1-256, 1-512, 1-1024, 1-2048, 1-4096. To each tube is added 0.5 c.c. bacillus emulsion, thus doubling each of the dilutions, so that instead of ranging from 1-2 to 1-4096, they range from 1-4 to 1-8192. In the twelfth tube are placed 0.5 c.c. salt solution and 0.5 c.c. bacterial emulsion to serve as a control of the emulsion and prevent error due to spontaneous clumping of the organisms. The tubes are placed in a water bath at 37° C. for one hour and then in the refrigerator over night. The clumping is observed with the naked eye, the clumps being visible and settling more rapidly than the bacterial emulsion. Should 1-512 of the final dilution show agglutination and 1-1024 fail to show it, the titer lies between these two, and it is advisable to set up a series of tubes 1-500, 1-600, 1-800, 1-900, 1-1000, and repeat. The same, of course, is true of the weaker dilutions, although beyond 1-1000 the scale is more easily placed in grades of 200 rather than 100. The preparation of such dilutions is illustrated as follows:

1	0.5 c.c. serum +	4.5 c.c. saline =	1-10 dilution
2	0.5 c.c. No. 1 +	12.0 c.c. saline =	1-25 dilution
3	0.5 c.c. No. 1 +	4.5 c.c. saline =	1-100 dilution
4	0.5 c.c. No. 2 +	4.5 c.c. saline =	1-200 dilution
5	0.5 c.c. No. 3 +	1.0 c.c. saline =	1-300 dilution
6	0.5 c.c. No. 2 +	5.5 c.c. saline =	1-350 dilution
7	0.5 c.c. No. 3 +	1.5 c.c. saline =	1-400 dilution
8	0.5 c.c. No. 2 +	8.5 c.c. saline =	1-450 dilution
9	0.5 c.c. No. 4 +	8.5 c.c. saline =	1-500 dilution

Should we wish to determine a titer between 1-200 and 1-500, dilutions 4-9 are placed, 0.5 c.c. in each of six tubes, 0.5 c.c. emulsion added to each, and in a seventh tube 0.5 c.c. saline and 0.5 c.c. emulsion as a control. The tubes are placed in the water bath and incubated as before. Similar protocols may be made if higher dilutions are required for the final test. Some workers prefer to set up primary dilutions of 1-10, 1-50, 1-100, 1-200, 1-500, 1-1000, 1-2000, 1-4000, but this has no particular advantages as compared with the primary titration outlined above.

Microscopic Titration.—The microscopic method may be employed with the same method of dilution and mixing, simply removing a drop for observation in a hanging drop preparation at the end of the period of incubation and examining with a 4-mm. lens. Another somewhat less accurate method is to place one loopful of each dilution on a coverslip and mix with a loopful of bacterial suspension, inverting the slip on a hollow ground slide, sealing with vaseline, incubating and reading the result. A still less accurate method is to place on coverslips or slides a row of loopfuls of salt solution, adding a loopful of serum to the first drop, mixing, transferring a loopful to the second

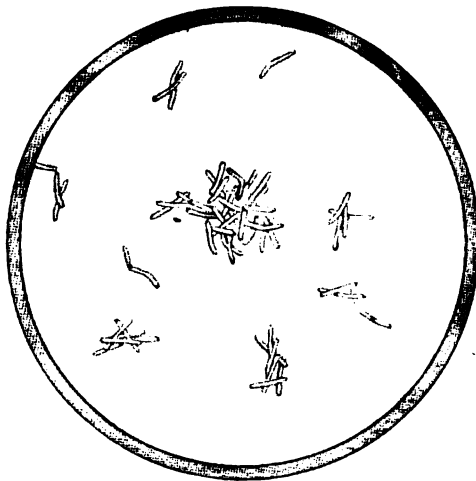


FIG. 10.—Microscopic drawing showing the agglutination of a suspension of bacillus typhosus by blood serum from a human case of typhoid fever, as seen in the Widal test.

drop and so on until the series of dilutions has been made, discarding a loopful of the last mixture and leaving one loopful of salt solution as a control, then adding to each drop a loopful of bacterial suspension. The slips or slides are inverted, sealed, incubated and read. In using slides, the trouble of sealing may be avoided by incubating in a moist chamber. The microscopic method is usually employed in the Widal test, the dilutions of patients' blood or serum being made by the same drop method, 1-20, 1-40, 1-80. Sometimes a drop of dried blood is used, this being laked and dissolved by a drop of water and then made up to the first dilution of 1-20 by the addition of nineteen drops of saline. Frequently twenty-four-hour broth cultures of the



FIG. 8.—The Wright tube for obtaining small quantities of blood serum.



FIG. 9.—Coiled pipette for taking up small quantities of fluids. Bubbling in the coil gives warning of the filling and prevents suction into the mouth. The tube may be made straight and plugged with cotton. Either may be used for withdrawing serum from the Wright pipette. Suction may be applied by the mouth directly or with a rubber tube or by means of a small nipple.

typhoid bacillus are employed as the emulsion. Clearer results, however, are obtained by collecting blood in Wright tubes (Fig. 8) and allowing the serum to separate for dilution, and then employing a salt solution suspension of a twenty-four-hour agar slant culture.

Specificity of Agglutinins—Group Reactions.—The specificity of the reaction may be shown by setting up dilutions of the anti-typhoid serum obtained from the immunized rabbit against suspensions of bacillus typhosus, bacillus paratyphosus (A or B), and bacillus coli communis. An illustrative protocol follows:

Typhoid immune serum	B. typhosus	B. paratyphosus A.	B. Coli
1-4	+	+	+
1-8	+	+	+
1-16	+	+	+
1-32	+	+	—
1-64	+	+	—
1-128	+	—	—
1-512	+	—	—
1-1024	+	—	—
1-2048	+	—	—
1-4096	—	—	—
Salt solution	—	—	—

This protocol illustrates two points, first, that the serum agglutinates its homologous bacteria in high dilutions, and second, that in strong concentrations it also agglutinates other organisms of the same group. Thus the specificity is not absolute throughout, but there is a "zone of absolute specificity," in this case between the

dilutions of 1-128 to 1-4096. The fact that the other two organisms are agglutinated is due to the phenomenon of "group reactions." In the same way, if an animal were immunized to bacillus coli the serum would agglutinate coli in high dilutions and typhosus in lower dilutions. The principle is also well shown in a table taken from Citron:

Agglutination of	Typhoid immune serum	Cholera immune serum
Against <i>B. typhosus</i>	1-2,000	1-10
Against <i>B. paratyphosus</i>	1-100	1-10
Against <i>B. coli</i>	1-25	1-10
Against <i>V. cholerae</i>	1-10	1-3,000

Absorption of Agglutinins.—If specific sera for paratyphosus and coli were interposed in the above diagram it would be seen that these sera would clump the homologous bacteria in high dilution, and the others of the group in only low dilutions. This indicates that in each serum we may assume there are several agglutinins, one for the homologous organism, a major agglutinin or main agglutinin, and one for each of the other organisms of the group, minor agglutinins or partial agglutinins. This statement may be accepted for the present, although the conception will be somewhat altered in the theoretical discussion. Castellani has shown that if the major agglutinin is absorbed by its homologous organism the minor agglutinins disappear also, but that if one or several of the minor agglutinins be absorbed by other members of the group of organisms the major agglutinin remains. In order to make this clear we shall first illustrate the process of absorption and then apply it to the group reaction. It is well known that an animal may be simultaneously immunized to two or more types of organisms; for example, bacillus typhosus and bacillus coli. The resulting serum may agglutinate typhosus in dilution of 1-4000 and coli in dilution of 1-1000. The absorption of the agglutinins may be shown as follows:

Prepare thick suspensions of bacillus typhosus and of bacillus coli communis by suspending the twenty-four-hour surface growth of three slant agar cultures in about 5 c.c. saline. This is done by placing 5 c.c. in the first tube, making the suspension, then transferring to the second tube, suspending that culture and repeating in the third tube. The typhoid emulsion is killed by heat of 56° C. for one hour and the colon by heat at 60° C. for one hour. Add to 1.5 c.c. serum an equal volume of thick suspension of dead bacillus typhosus and in another tube place 1.5 c.c. serum with an equal volume of thick suspension of dead colon bacilli. The tubes are marked A and B. After mixing the emulsion of bacilli and serum the tubes are incubated at 37° C. and placed in the ice-chest for twelve hours. The tubes are centrifuged and the supernatant fluid pipetted off. The bacteria are resuspended and the suspensions diluted with salt solution about 1-20 or more, in order that agglutination may be easily observed. The supernatant fluid represents a 1-2 dilution of the original serum. Place 0.5 c.c. each into test tubes and add 4.5 c.c. saline, making a dilution of 1-20, well under the titer of the serum. Of the diluted fluid A which has been absorbed by typhosus place 0.5 c.c. in a series of two tubes and add 0.5 c.c. thin emulsion of colon. After incubation the first tube will show no agglutination, and the second tube containing colon, whose agglutinin has not been absorbed, will show agglutination. Conversely place 0.5 c.c. diluted fluid B in a series of two tubes, and add in order 0.5 c.c. thin

emulsion of typhosus and 0.5 c.c. thin emulsion colon. After incubation only tube 1 shows agglutination, because the colon agglutinins have been absorbed. The protocol of this experiment with the controls follows:

SERIES A (ABSORBED BY TYPHOSUS)

1. Fluid A 0.5 c.c. + 0.5 c.c. typhosus = no agglutination.
2. Fluid A 0.5 c.c. + 0.5 c.c. colon = agglutination.

SERIES B (ABSORBED BY COLON)

3. Fluid B 0.5 c.c. + 0.5 c.c. typhosus = agglutination.
4. Fluid B 0.5 c.c. + 0.5 c.c. colon = no agglutination.

CONTROLS

5. Saline 0.5 c.c. + 0.5 c.c. typhosus = no agglutination.
6. Saline 0.5 c.c. + 0.5 c.c. color = no agglutination.

This experiment shows only the essentials of the specific absorption. It may be further elaborated by making a series of dilutions of the treated serum so as to show the fact that the titer is essentially unimpaired.

Tube	Fluid A 0.5 c.c.	Typhosus emulsion	Result
1	1-8	0.5 c.c.	+
2	1-16	0.5 c.c.	+
3	1-32	0.5 c.c.	+
4	1-64	0.5 c.c.	+
5	1-128	0.5 c.c.	+
6	1-256	0.5 c.c.	+
7	1-512	0.5 c.c.	+
8	1-1,024	0.5 c.c.	+
9	1-2,048	0.5 c.c.	+
10	1-4,096	0.5 c.c.	—
11	1-8	Colon emulsion	—
12	1-16	0.5 c.c.	—
13	1-32	0.5 c.c.	—
14	1-64	0.5 c.c.	—
15	Saline	0.5 c.c.	—
16	Saline	Typhosus 0.5 c.c.	—

At the same time set up tubes as follows:

Tube	Fluid B 0.5 c.c.	Colon emulsion	Result
1	1-8	0.5 c.c.	+
2	1-16	0.5 c.c.	+
3	1-32	0.5 c.c.	+
4	1-64	0.5 c.c.	+
5	1-128	0.5 c.c.	+
6	1-256	0.5 c.c.	+
7	1-512	0.5 c.c.	+
8	1-1,024	0.5 c.c.	—
9	1-8	Typhosus emulsion	—
10	1-16	0.5 c.c.	—
11	1-32	0.5 c.c.	—
12	1-64	0.5 c.c.	—
13	Saline 0.5 c.c.	0.5 c.c.	—
14	Saline 0.5 c.c.	Colon emulsion 0.5 c.c.	—

This experiment shows that the process of absorption removes only the specific agglutinin and leaves the other agglutinin unchanged. As a matter of practical fact, the typhoid agglutinin remains unchanged, but the colon agglutinin may be somewhat reduced in titer, perhaps to 1-800 or even as low as 1-300. In a combined serum of this sort with the typhoid agglutinin of high titer, part of the agglutinin for colon is the result of a typhoid minor agglutinin which is removed by absorption with typhosus, thus reducing the

colon titer. The primary colon titer of 1000 would have a very low content of minor agglutinin for typhosus, the removal of which would leave the primary titer for typhosus practically unchanged after absorption with colon bacilli.

The differences of absorption of major and minor agglutinins may be illustrated by the use of a typhosus immune serum. We may use, for illustration, as closely related organisms bacillus typhosus and bacillus paratyphosus B. Preliminary titration of the serum is carried out as usual against bacillus typhosus and bacillus paratyphosus B. Let us suppose that the serum shows a titer of 1-4096 for typhosus and 1-512 for paratyphosus B. Thick emulsions of typhosus and para B are made as described in the previous experiment, killed by 56° C. for one hour and mixed in equal volume with 1.5 c.c. serum, incubated for one hour and refrigerated for twelve hours, then centrifuged and the fluid pipetted off. The experiment with the results may be illustrated in the following protocol:

Tube	Fluid A 0.5 c.c. (absorbed by typhosus)	Typhosus emulsion	Result
1	1-16	0.5 c.c.	—
2	1-32	0.5 c.c.	—
3	1-64	0.5 c.c.	—
4	1-128	0.5 c.c.	—
5	1-256	0.5 c.c.	—
6	1-512	0.5 c.c.	—
7	1-1,024	0.5 c.c.	—
8	1-2,048	0.5 c.c.	—
Tube	Fluid A 0.5 c.c. (absorbed by typhosus)	Para B emulsion	Result
10	1-16	0.5	—
11	1-32	0.5	—
12	1-64	0.5	—
13	1-128	0.5	—
14	1-256	0.5	—
15	Saline 0.5 c.c.	Typhosus 0.5 c.c.	—
16	Saline 0.5 c.c.	Para B. 0.5 c.c.	—
Tube	Fluid B 0.5 c.c. (absorbed by Para B)	Typhosus emulsion	Result
17	1-16	0.5 c.c.	+
18	1-32	0.5 c.c.	+
19	1-64	0.5 c.c.	+
20	1-128	0.5 c.c.	+
21	1-256	0.5 c.c.	+
22	1-512	0.5 c.c.	+
23	1-1,024	0.5 c.c.	+
24	1-2,048	0.5 c.c.	+
25	1-4,096	0.5 c.c.	—
		Para B emulsion	
26	1-16	0.5 c.c.	—
27	1-32	0.5 c.c.	—
28	1-64	0.5 c.c.	—
29	1-128	0.5 c.c.	—
30	1-256	0.5 c.c.	—
31	Saline 0.5 c.c.	0.5 c.c.	—
32	Saline 0.5 c.c.	Typhosus 0.5 c.c.	—
	Untreated serum		
33	1-2,048	Typhosus 0.5 c.c.	+
34	1-256	Para B. 0.5 c.c.	+

It will be seen from these protocols that absorption by the major agglutigen, *bacillus typhosus*, removes both the major and minor agglutinins, and that absorption by the minor agglutigen removes only the minor agglutinin, although it is true that even though the titer of the major agglutinin is not reduced it may agglutinate in smaller clumps.

Inhibition Zones.—It is sometimes found that in powerful agglutinins there is an "inhibition zone" in the more concentrated dilutions. Thus a serum may agglutinate as follows:

Tube	Serum dilution	Result
1	1-10	—
2	1-100	+
3	1-1,000	++++
4	1-2,000	++++
5	1-4,000	++
6	1-6,000	+
7	1-8,000	—

This phenomenon is somewhat more frequently observed in sera that have been preserved for a considerable time in the moist state. If a serum with a titer of 1-1000, which originally showed agglutination in all dilutions up to 1000, is preserved and after several months titrated again, it may fail to agglutinate in 1-10, may agglutinate only weakly in 1-100, and completely in 1-500. If the tube containing 1-10 dilution is centrifuged, the supernatant fluid drawn off, the bacteria again suspended and placed with the serum in dilution of 1-500, there is no agglutination. The same is true if these treated organisms are placed in contact with a fresh agglutinating serum. The same phenomenon is obtained if the serum first used is a fresh one of high titer with an inhibition zone, and the bacteria are removed from the low dilutions in which they have failed to agglutinate. The bacteria have become inagglutinable by treatment with the serum in these concentrations. Similarly, heating an agglutinating serum to 65° to 70° C. destroys its agglutinating properties, but if it is added to bacteria they become inagglutinable when treated with fresh active serum. This phenomenon is strictly specific and operates only in the presence of the homologous organism. This peculiar character of agglutinins has been closely linked with the Ehrlich conception of immune bodies and is explained as due to the presence in sufficient concentration of "agglutinoids." The term agglutinoid is applied to that part of the agglutinin which has a specific binding affinity for the cell, but has been deprived of the thermolabile and more easily destructible fraction which has the power of producing clumping. This explanation will be discussed more in detail in the general discussion of agglutinins.

The influence of heat on agglutination has been studied extensively. As has been indicated, heat will destroy agglutinins, but certain agglutinins are destroyed by degrees of heat which fail to destroy others. Most agglutinating sera are rendered inactive at 60° to 65° C., but anti-plague agglutinin is destroyed at 56° C.,

whereas others do not disappear until 80° C. has been reached. Wells states that "purified typhoid agglutinin may resist 80 to 90 degrees." Agglutinins cannot be reactivated by the addition of fresh serum, even though the temperature may have been adjusted so that the agglutinoïd remains.

A simple experiment for the demonstration of the influence of heat on agglutinins is as follows: The typhoid immune serum, the production of which has been described above, and also the killed typhoid suspension may be used. In each of three tubes place 0.5 c.c. serum diluted 1-10, and into a fourth tube 0.5 c.c. salt solution. Tube 1 is heated in a water bath at 56° C. for one-half hour, tube 2 heated at 70° to 75° C. for one-half hour, and tubes 3 and 4 kept at room temperature. After cooling tubes 1 and 2, add 0.5 c.c. bacterial emulsion to each tube and incubate for one hour at 37° C. Agglutination will not occur in tube 2, the serum having been heated to 70° to 75° C., nor in the control tube with saline. The unheated serum and the serum heated to 56° C. will agglutinate powerfully. It will be found also that the addition of 0.1 c.c. fresh guinea-pig serum (complement) to tube 2, and subsequent incubation will fail to produce agglutination.

It is of interest to note that the degree of concentration of serum has some influence on the degree of heat necessary for destruction. For example, Koeckert in this laboratory found that normal undiluted iso-hemagglutinins are destroyed at 65° to 66° C. for thirty minutes, but that in high dilutions they are destroyed at 62° C. for thirty minutes.

The influence of electrolytes on the phenomenon of agglutination is of considerable importance from the theoretical point of view because of the resemblance to flocculation of colloidal suspensions. Bordet, who discovered this fact, compared the reaction to the throwing down of the alluvial matter in rivers as the fresh water meets the salt water of the ocean. By previously dialyzing the salts out of the bacterial suspension and the specific serum he showed that agglutination would not occur, but that if the mixture was salted in proper concentration the reaction would take place. It is possible, however, to agglutinate bacteria by certain concentration of salts, particularly of the heavy metals, but such concentration is always much stronger than is necessary for salting, as described in the Bordet experiment.

The demonstration of the influence of salts may be seen in the following experiment, taken from Zinsser, Hopkins and Ottenberg. For this experiment the killed typhoid suspension and the anti-typhoid serum as employed in previous experiments may be used. "Place in each of two centrifuge tubes with pointed tip 2.0 c.c. of the suspension. To tube A add 2.0 c.c. of agglutinating serum diluted 1-50. To tube B add 2.0 c.c. distilled water. Allow the tubes to stand at 37° C. for thirty minutes. Centrifugalize the tubes at high speed until the supernatant fluid is clear." Pipette off the fluid and "to the washed sediments add 2.0 c.c. distilled water and draw the mixture repeatedly in and out of the capillary pipette in order to break up the clumps and obtain an even suspension. Set up the following tests in agglutination tubes:

1	Sediment A 0.5 c.c.	Distilled water 0.5 c.c.
2	Sediment A 0.5 c.c.	10 per cent NaCl 0.09 c.c.	Distilled water 0.5 c.c.
3	Sediment A 0.5 c.c.	0.8 per cent. CuSO_4 0.02 c.c.	Distilled water 0.5 c.c.
4	Sediment B 0.5 c.c.	0.8 per cent. CuSO_4 0.02 c.c.	Distilled water 0.5 c.c.
5	Sediment B 0.5 c.c.	0.8 per cent. CuSO_4 0.1 c.c.	Distilled water 0.5 c.c.
6	Sediment B 0.5 c.c.	Distilled water 0.5 c.c.

"The tubes are placed in the water bath at 37° C. for one hour and then observed. Tubes 2, 3 and 5 should show agglutination." In tube A the bacteria have been 'sensitized' with the immune serum, and after the clumps have been broken up are ready again for clumping under proper conditions. In tube 1 the addition of distilled water does not provide the essential conditions, but in tubes 2 and 3 the addition of electrolytes favors the reaction. In tube B the bacteria have not been sensitized, but of the tubes 4, 5 and 6, the concentration of the copper sulphate is such as to induce clumping in itself, a phenomenon frequently seen in certain concentrations of salts of the heavy metals, such as zinc, lead and mercury.

Influence of Hydrogen Ion Concentration.—It has been shown by Michaelis and others that bacteria may be agglutinated by providing a proper hydrogen ion concentration, and it was hoped that this might provide a means of rapid identification of organisms. Proteins, for example, have a specific and constant optimum concentration of H ions for their precipitation. In the case of bacteria it was shown, for example, that *bacillus typhosus* was agglutinated by a hydrogen ion concentration of 4 to 8×10^{-5} , whereas *paratyphosus* requires 16 to 32×10^{-5} , colon bacilli not being agglutinable by this method. It has been shown, however, that this differentiation is not so sharp as was at first supposed, that different strains show considerable irregularity, and that there is overlapping of one species with another. A combination of serum and acid agglutination has shown that bacteria sensitized by serum can be more readily agglutinated than are non-sensitized bacteria. The specific characters of bacterial proteins are probably due to such a slight variation in the arrangement of the molecular structure that a satisfactory differentiation by changes in hydrogen ion concentration is not at present feasible. Eisenberg has recently studied the problem with 584 races of bacteria, of which 537 were of the colon-typhoid group, and found no differential diagnosis possible with the acid agglutination method. He also found flocculation with salts of the heavy metals extremely variable.

The Mechanism of Agglutination.—The data given in the preceding paragraphs outline the most important phases of the phenomenon of agglutination, and any discussion of the mechanism of the process must be based on these fundamentals. The chemical nature of the agglutinin is, of course, closely combined, if not identical, with the protein of the cells, but is in no sense dependent for its activity on the existence of life within the cell. Agglutinogens are not destroyed by mild concentrations of formalin, phenol, heat, or ultra-violet rays which are sufficient to destroy the life of the cell itself. They pass through dialyzing membranes more rapidly than do the agglutinins, and therefore are probably made up of smaller molecules. That they pass through collodion sacs can be shown by implanting such sacs, filled with killed typhoid organisms, in the peritoneal cavity of rabbits and observing the development of agglutinins in their blood; an observation which has been confirmed by Reimann in this laboratory. Old broth cultures contain in the fluid agglutinogens which may neutralize agglutinins and which may

serve also to produce agglutinins upon injection. Thus it would appear that agglutinogens are bodies of small molecular size capable of slow diffusion and almost certainly protein, although Stuber maintains that they are of fatty nature. The influence of heat on agglutinogens has been carefully studied by Joos, who concluded that the agglutinogen consists of relatively thermolabile and thermostable constituents (the dividing line being 60° to 62° C.) which induce the formation of separate agglutinins. The thermostable fraction resists heat up to 165° C., is soluble in alcohol, and does not give protein reactions, whilst the thermolabile fraction gives all the protein reactions. This work is more fully discussed subsequently.

Alterations of Agglutinability.—Of considerable interest in connection with agglutinogens is the alteration of agglutinability of the cell. This probably is more closely associated with the cell as such than with the agglutinin. If bacteria are heated above 65° C. they are not agglutinable by specific immune sera, but can absorb agglutinin from the sera. Organisms freshly isolated from cases of infectious disease often show similar reductions of agglutinability, but recover it after prolonged growth on artificial media. This is likely to be true in the case of "carriers," and Welch has referred to it as a quasi-immunity which the bacteria themselves have acquired by acting against the immune bodies of the host, an immunity, however, which the organisms lose on living in the environment of the artificial culture media. Such inagglutinability may be produced artificially by growing the bacteria on media containing a specific immune serum, heated to destroy any bacteriolytic influence. In a personal communication to us M. Cooper has stated that the presence of capsules about bacteria serves to establish a quasi-immunity for the organisms against antibodies, and that such capsules appear after cultivation in immune sera. This peculiar phenomenon is explained on the Ehrlich theory by assuming that the bacteria are practically exhausted of receptors. Nevertheless, such inagglutinable bacteria upon injection into animals lead to the production of agglutinins for agglutinable strains, but not for inagglutinable strains. It has also been assumed that they are saturated with agglutinoid, but in America, at least, the Welch theory has been given wide acceptance as an important philosophical conception. Not only may agglutinability be altered, but different strains of an organism show natural differences in agglutinability. For example, Cole has shown that against a specific agglutinating serum five strains of pneumococcus showed titers of 1-4000, 1-4500 (2), 1-7000, and 1-8000. These are not "types" of a species but strains, and show no specific agglutinability for sera produced by the strain in question.

The Nature of Agglutinins.—The chemical study of the agglutinins shows that, like antitoxins, they are precipitated out of the serum in the globulin fraction, and so far they have not been further purified. They pass through filters less readily than their antigens, and therefore have a larger molecular structure. Pepsin

digestion destroys the agglutinins fairly readily, but trypsin acts more slowly. Alkalies even when dilute are destructive, but acids operate less actively. They are absorbed by charcoal. They are not thrown down in the precipitate formed by specific precipitating sera. The influence of heat on agglutinins has been the subject of much study. The work of Joos was conducted with both agglutigen and agglutinin. As mentioned above, he demonstrated the presence in the bacterial antigen of a thermolabile A agglutigen and a thermostable B agglutigen, the dividing line being 60° to 62° C. The injection of heated antigen (B agglutigen) gives rise to the formation of B agglutinin, which in contrast to the antigen is destroyed by heat of 60° C., but reacts with both A and B agglutinogens. The injection of the unheated bacilli containing both A and B agglutigen leads to formation of both agglutinins, but the B agglutinin can be removed by heat leaving the thermostable A agglutinin, which reacts only with the A agglutigen. The essentials of this work have been confirmed, although Scheller working with bacillus typhosus found that the B agglutinin is reduced in titer but not completely destroyed at 60° to 62° C. Scheller showed further that the heated bacteria (B agglutigen) absorb agglutinins from the sera more readily than do unheated bacteria, and that they give the highest titers with the serum.

According to the Ehrlich scheme, agglutinins have a haptophore or combining group and a zymophore group which causes the agglutination. This zymophore is killed by heat and deteriorates on long standing to form the agglutinoid (or agglutinin free from zymophore), which has combining but not agglutinating power. Thus in the side-chain theory the agglutinins (and precipitins) differ from the theoretical simplicity of the antitoxins and constitute the receptors of the second order.

The Physical Basis of Agglutination.—The mechanism of agglutination is such that the reaction takes place in constant proportions, thus likening it to a simple chemical reaction. The reaction is reversible, however, in that simple shaking, the use of organic and inorganic acids and acid salts, as well as alkalies and heat of 70° to 75° C., can break the clumps into cell units; but after this separation fresh agglutinating serum cannot operate again. It has been shown further that agglutinins can be separated from bacteria-agglutinin combinations by the electric current; therefore, the agglutinins are not destroyed by the union with the bacteria. Many of the older workers believed that the reaction occurred because of changes in the outer layers or ectoplasmic substance of the cells. Gruber at first maintained that a substance, glabrificin, was taken from the serum by the cells which made their outer surfaces sticky and caused adhesions when their motility brought the bacteria in contact with one another. Malvoz and others held that the reaction depended upon the entanglement of the flagella of the bacteria. Neither of these ideas is consistent with the fact that non-motile

bacteria and other cells are subject to agglutination, but no definite proof is at hand to show that the ectoplasmic substance is not of considerable importance. The influence of salts on agglutination lends much support to the conception that agglutination is a colloidal phenomenon. As has been indicated above, the presence of electrolytes is essential to the reaction, but salts, acids, and salts of heavy metals, if present in sufficient concentration, may of themselves produce agglutination. On the other hand, salts in strong concentration serve to prevent the action of agglutinin. When bacteria have absorbed agglutinin, very small amounts of salt serve to bring about agglutination. If a suspension of bacteria and an agglutinating serum are each dialyzed free of salt and the two mixed, the bacteria absorb agglutinin. This is shown by the fact that the supernatant fluid after centrifugalization is free of agglutinin, but agglutination occurs on addition of salt. Bordet interpreted the phenomenon of agglutination as having two phases, first that of sensitization of the bacteria by the agglutinin, and second, that of agglutination of these agglutinin-bacteria by the salt. It may be stated in other terms that the bacteria are primarily suspensions of protected colloids which are so altered by the agglutinin that they become unprotected and precipitable by salts, or that they become more permeable for electrolytes. In fact, it has been shown that sensitized bacteria take up salts more readily than unsensitized. The similarity of bacteria to protected colloids is also borne out by Porges, who showed that while encapsulated organisms are inagglutinable, the solution of their capsules by heating in weak acid renders the bacteria agglutinable. Bacteria carry electro-negative charges and move toward the anode, whereas agglutinins are electro-positive. The sensitized bacteria are agglutinated by the current between the poles, although the sensitized bacteria move slowly toward the anode. The small amount of salt necessary for agglutination further supports the influence of electrical charge and thus furnishes further analogy with colloidal precipitation. Neisser and Friedemann have studied the similarities of agglutination and colloidal precipitation and offer much in support of such analogy. Two protocols may serve to show the importance of their work, one dealing with the so-called sensitization and the other with inhibition zones. Just as salt influences agglutinin and agglutinogen, so may it influence mastic and gelatin solutions, as may be seen in the following experiment:

10% NaCl Sol.	1.0 c.c. mastic (1-10 original emulsion) diluted to 3.0 c.c.	1.0 c.c. mastic + 0.0001 c.c. 2% gelatin sol. and diluted to 3.0 c.c.
1.0 c.c.	+	+
0.5 c.c.	—	+
0.25 c.c.	—	+
0.125 c.c.	—	+
0.05 c.c.	—	—
0.025 c.c.	—	—

Furthermore, they offer a protocol showing the similarity between the reaction of colloidal iron hydroxide upon mastic emulsions and the agglutination phenomenon in reference to inhibition zones. It will be seen that stronger concentrations of the iron hydroxide fail to precipitate, thus simulating the action of strong concentrations of an agglutinating serum of high titer or of an old serum. The protocol follows:

Colloidal iron hydroxide	Mastic emulsion	Result
1.0	1.0 c.c.	0
0.5	1.0 c.c.	0
0.25	1.0 c.c.	0
0.1	1.0 c.c.	++
0.5	1.0 c.c.	++++
0.025	2.0 c.c.	++++
0.01	1.0 c.c.	++++
0.005	1.0 c.c.	++++
0.0025	1.0 c.c.	++
0.001	1.0 c.c.	0

This latter protocol is of significance not only in relation to agglutination, but is of importance also in connection with the Neisser-Wechsberg phenomenon of complement-deviation (not fixation) discussed in connection with bacteriolysis. As Zinsser says, "it seems to be a universal fact governing the union of colloidal substances, that definite quantitative proportions must be maintained in order to lead to reaction, this being, possibly, explicable on the basis that actual union can take place only after disturbance of the electrical balance which keeps the particles apart." The assumption that agglutinoids have an important bearing on the presence of inhibition zones is not necessary if we accept the colloidal nature of agglutination. This does not entirely controvert the existence of altered agglutinin with a binding power for agglutigen.

Not only may salt-free bacteria-agglutinin combinations be agglutinated by salts but, as Friedberger has shown, certain organic substances, such as dextrose and asparagin, serve also to produce agglutination in such salt-free mixtures. These substances do not dissociate in solution as do salts, and therefore produce no electric phenomena. This fact presents a certain objection to the final acceptance of the colloidal theory of agglutination, but it is possible that the mechanism in this instance is of a nature different from that of the immunological process, and certainly the great mass of evidence is in favor of the reaction of agglutination being of colloidal nature.

Nothing has been definitely brought forward in the physico-chemical examination of agglutination to explain specificity, except the fact previously indicated, that variations of hydrogen ion concentration have a relatively specific action on bacteria. As is known, the definite identification of bacteria by this method has not been satisfactory. The specificity of immune serum agglutina-



FIG. 11.—The nipple pipette for making mixtures of fluids and bacterial suspension. The pencil mark is seen a short distance above the tip.

tion is also a relative matter, as is shown in the group reactions, and if electric phenomena play a part in specificity they are more delicate than can be demonstrated by present chemical or electrical methods. Bordet, who laid no emphasis on the electrical reactions, thought that the process of sensitization of bacteria by agglutinins is in essence a denaturing of the bacterial proteins, and that the specificity of the process depends on the degree of denaturation.

The Dreyer Test.—The Widal test has been described (page 85). This test has been of the greatest service in the diagnosis of typhoid and paratyphoid fevers but the introduction of vaccination on a large scale has reduced the value of the test as a diagnostic sign of actual disease, because vaccinated individuals give a positive test. Dreyer studied the course of agglutination in typhoid and paratyphoid fevers, and found that the agglutinative titer of the blood follows, during the course of the disease, a fairly regular curve, increasing to the third week and then declining. Although the titer may be higher at the beginning of the disease in vaccinated individuals than in others, the titer follows the same general curve. Of more importance is the differentiation between typhoid and other infections in the vaccinated. This has been of the utmost importance in the World War in distinguishing between febrile disease, such as trench fever or malaria, and typhoid or paratyphoid. The test is made by the macroscopic method for agglutination, and must be repeated at weekly intervals in order to determine the curve of agglutinins. Not infrequently the first test may show a titer so much higher than occurs after vaccination that a presumptive diagnosis is justifiable. Under war conditions the transfer of patients often made it necessary to perform the tests in several different laboratories, and to provide for this the Oxford Standards Laboratory prepared emulsions of the bacilli for distribution. For this purpose the organisms were grown for twenty-four hours in pepton veal broth, then shaken well and 0.1 per cent. formalin (40 per cent. formaldehyde) added. The culture was stored at 2° C. and shaken frequently during four or five days. At the end of this time it was usually sterile. It was then diluted to standard opacity by means of salt solution, to which was previously added 0.1 per cent. formalin. It was further standardized as to agglutinability and labeled with a factor so as to provide means whereby tests in different laboratories could be estimated on the same basis.

The blood for the test can be obtained in a Wright tube, but it is preferably taken from the cubital vein into a centrifuge tube, so as to provide a fairly large amount of serum. In order to make the method applicable in laboratories where graduated pipettes are not available, Dreyer made all the dilutions with a nipple pipette of drawn-out glass tubing similar to that illustrated in Fig. 11, except that the drawn-out part is wider and shorter. Three rows of 7 x 75 mm. test tubes are then set up and further dilutions made according to the following scheme:

Tube	Water	Serum	Bacterial suspension	Dilution equals
1	.0 drop	10 drops	15 drops	1-25
2	5 drops	5 drops	15 drops	1-50
3	8 drops	2 drops	15 drops	1-125
4	9 drops	1 drop	15 drops	1-250
5	10 drops	0 drop	15 drops	control

The three rows of tubes are set up so as to use suspensions in each row of bacillus typhosus, paratyphosus A, and paratyphosus B. The dilutions may be carried further if necessary. The tubes are incubated in a water bath at 55° C. for two hours, are read immediately, and, if desired, again after twenty-four hours in the refrigerator. The standard method of Dreyer may be adapted to other methods of dilution and incubation, but must be the same in the study of every case.

In unvaccinated individuals agglutination in a dilution of 1-25 against bacillus typhosus justifies suspicion, and if marked in dilution of 1-50 is nearly always diagnostic. Browning offers the following table as indicating positive reactions in each of the diseases indicated.

Organism	Serum dilution
B. typhosus	1-100
B. paratyphosus A.	1-50 (or even lower 1-20)
B. paratyphosus B.	1-200

These criteria are not applicable to vaccinated persons or those who have previously had typhoid or paratyphoid fever. Martin and Upjohn examined seventy-five persons from seven to fourteen months after typhoid vaccination and found that the serum of two-thirds agglutinated bacillus typhosus in serum dilutions of 1-200, and that of one-tenth agglutinated in dilutions of 1-800. These are higher levels than are usually reached by unvaccinated persons during the course of the disease. Vaccination with typhoid vaccine produces minor agglutinins for para A and B, but in very low concentration. Triple vaccines produce agglutinins for para A and B, but rarely in dilutions exceeding 1-50 or 1-100. The following chart, taken from Mackie and Wiltshire, as quoted by Browning, illustrates the change in titer of blood serum in the course of infection with bacillus paratyphosus A.

	Serum dilutions					
	1-50	1-100	1-200	1-500	1-1000	1-2000
Fourth or fifth day of illness:						
B. typhosus.....	+	-	-			
B. paratyphosus A.....	+++	+	-			
B. paratyphosus B.....	-	-	-			
Thirteenth day of illness:						
B. typhosus.....	+++	++	+	-	-	-
B. paratyphosus A.....	++++	++++	++++	++++	+++	++
B. paratyphosus B.....	++	-	-	-	-	-

The first test in this patient was strongly suggestive, since it is rare in a vaccinated individual to find the titer for either para A or B to exceed that of typhosus. In our experience typhoid in the vaccinated is likely to show titers in the first week of 1-500 for typhosus, 1-100 for para A, and 1-50 for para B; toward the end of the second week they are likely to be, respectively, 1-2500, 1-750, 1-250; and in the third week 1-3000 or higher for typhosus with slight increases for para A and B. The titers then subside. It will be noted that infection increases not only the major but also the minor agglutinins.

Space does not permit a complete discussion of the results of the test, but it may be said that a positive Dreyer test indicates the presence of some form of enteric fever. If, however, the isolation of organisms from the stools indicates the nature of the disease the test may sometimes mislead. For example, we have found paratyphosus B in the stools of a patient whose serum titer curve indicated the presence of a para A infection. The test should go hand in hand with careful clinical study and bacteriological examination of the blood, feces, and urine.

Hemagglutinins.—The agglutination of blood-cells and other body cells follows the same general principles laid down for bacterial agglutinins. In the case of agglutinins for red blood-corpuscles the name hemagglutinins has been adopted. These may be divided into auto-hemagglutinins, iso-hemagglutinins, and hetero-hemagglutinins. The auto-hemagglutinin is contained in the same blood as the cells it agglutinates, but certain factors operate to prevent agglutination in the living body. For example, Rous and Robertson have shown the presence in rabbits, which had received repeated small blood transfusions, of an auto-hemagglutinin which operates at temperatures lower than that of the animal, but on raising the temperature to 38° to 40° C. the clumps break up and a homogeneous emulsion results. The same workers also demonstrated the presence of auto-agglutinins in rabbits subjected to repeated withdrawal of small quantities of blood. It has been stated that this phenomenon may also occur in acquired hemolytic jaundice (Hayem-Widal type), pernicious anemia, malaria, and other diseases, but more recent studies tend to contradict this statement. Hornby states that auto-hemagglutinins have been demonstrated frequently in animals infected with trypanosomes. Hetero-agglutinins were discovered by Creite and Landois, who noted that the serum of certain animals produced agglutination when brought in contact with the cells of certain other species; for example, the serum of the goat and the erythrocytes of rabbit, man, or pigeon. Bordet discovered in the course of his studies on hemolysins that if an animal is immunized with the erythrocytes of another species, the blood serum will contain not only hemolysin, but also hemagglutinin for the cells used in immunization. Thus we have to consider normal hetero-hemagglutinins and immune hetero-hemagglutinins. Such normal antibodies are present in low titer, but immune agglutinins of this sort may be induced up to titers of several thousand. The methods employed for the production of such agglutinins are the same as those for producing hemolysis and will be considered under that subject. The determination of the titer of hemagglutinative sera is by essentially the same methods as for bacterial agglutinins, save that the cells are washed as for experiments in hemolysis, and usually a fixed percentage emulsion of cells is employed. The influence of heat and other physical agents, as well as chemicals, is much the same as for hemolysins (see page 115).

Iso-hemagglutinins, Classification.—Iso-hemagglutinins are those which exist in certain members of a species for cells of certain other members of the same species. Although iso-hemagglutinins may somewhat rarely occur in lower animals, they appear with great regularity in human blood. They were discovered in 1906 by Landsteiner and Shattock, working independently. Landsteiner, by a study of the interaction of sera and corpuscles, classified all human bloods in three groups and determined that the property of iso-agglutination is normal to man and does not vary under pathological conditions. Hektoen noted in 1907 that the three groups do not include all individuals, and in the same year Jansky published the classification in four groups. This was confirmed by Hektoen and subsequently adopted by Ottenberg. Moss, in 1910, without knowledge of Jansky's work, also found that it is necessary to divide bloods into four groups in order to include all individuals, but unfortunately employed a system of numbering the groups the opposite of that of Jansky. Because of the priority of Jansky's system and its important support by Hektoen and by Ottenberg and others, we prefer to use it rather than that of Moss. Groups I and IV are transposed in the two systems but Groups II and III remain the same, hence, groups are transposable from one basis to the other. The groups are not present at birth, but become established at about the end of the first year of life and remain constant thereafter; they are heritable according to the Mendelian law. Disease does not change the group of an individual, although, according to some of our experiments, it seems possible that the agglutinin titer may be somewhat reduced by prolonged disease. Jansky included in Group I those bloods whose sera agglutinate cells of all other groups and whose cells are not agglutinated by any sera; Group IV is the reciprocal of Group I in that the sera agglutinate no cells, but the cells are agglutinated by sera of all the other groups. Groups II and III are reciprocals of each other and occupy intermediate positions between Groups I and IV. This may be rendered clearer by the following table:

Group I.	Sera agglutinates cells II, III and IV. Cells agglutinated by no sera.
Group II.	Sera agglutinates cells III and IV. Cells agglutinated by sera I and III.
Group III.	Sera agglutinates cells II and IV. Cells agglutinated by sera I and II.
Group IV.	Sera agglutinates no cells. Cells agglutinated by sera I, II and III.

The following chart presents the classification graphically; the + sign indicates agglutination:

		JANSKY CLASSIFICATION			
		Sera			
		I.	II.	III.	IV.
Cells.	I.	—	—	—	—
	II.	+	—	+	—
	III.	+	+	—	—
	IV.	+	+	+	—

Inasmuch as the Moss classification has been widely adopted we include the chart of that system so as to show the relation of the two systems of grouping:

MOSS CLASSIFICATION					
		Sera			
Cells.	I.	I.	II.	III.	IV.
	II.	—	+	+	+
	III.	—	—	+	+
	IV.	—	—	—	—

It is of the utmost importance that when the groups are determined in any individual the method of classification should be clearly stated.

The incidence of the groups varies somewhat, according to the figures of different investigators, and there is probably a factor of error due to "random sampling," in spite of the large number of individuals examined. Selected figures follow, according to the Jansky classification:

		Groups							
		I.		II.		III.		IV.	
Von Dungern									
Hirschfeld	36.	per cent.	47.	per cent.	11.	per cent.	6.	per cent.	
Moss	43.	per cent.	40.	per cent.	7.	per cent.	10.	per cent.	
Olmstead	46.	per cent.	39.	per cent.	13.	per cent.	2.	per cent.	
Karsner	46.2	per cent.	42.4	per cent.	8.3	per cent.	3.1	per cent.	
Koeckert	43.	per cent.	38.5	per cent.	12.5	per cent.	6.	per cent.	
Average	42.84	per cent.	41.38	per cent.	10.36	per cent.	5.42	per cent.	

The table shows that about four-fifths of all individuals fall in Groups I and II, about equally divided between the two groups, the next most frequent being Group III, and the least frequent being Group IV.

Characters of Iso-hemagglutinins.—The iso-hemagglutinins are neither filterable nor dialyzable, and are destroyed by heat of 62° to 66° C. for thirty minutes, depending on concentration, *i.e.*, the agglutinins in high dilutions (1-32, 1-64) disappear at 62° C., and in the undiluted sera at 65° to 66° C. They are present in transudates and exudates as well as in the plasma and serum, the serum showing a greater concentration than the plasma. In serum the titer is usually between 1-16 and 1-32, although it may be as low at 1-2, and has been reported as high as 1-320, irrespective of group. There is variation of agglutinin content and probably of agglutinability of cells at different times in the same individual.

The fact that a blood contains an iso-agglutinin does not necessarily mean that it will similarly dissolve corpuscles, but the converse is true; namely, that if a serum shows iso-hemolytic properties it is also iso-hemagglutinative; the group relationship prevails in both agglutination and hemolysis. In fact, agglutination always precedes hemolysis. In spite of this generally accepted view, Kolmer claims recently to have demonstrated the presence of iso-hemolysins independent of iso-agglutinins.

LAW OF AGGLUTININS AND PRECIPITINS

The Mechanism of Iso-hemagglutination.—Numerous theories have been offered, of which we present that of Landsteiner. It has recently received support in this laboratory by the painstaking specific absorption experiments of Koeckert. Landsteiner considers that the division into four groups depends upon the presence, differently distributed in bloods, of two agglutinins, a and b, and two agglutinogens, A and B. The distribution of these may be tabulated as follows (Jansky classification):

Group	Agglutinins (serum)		Agglutinogens (cells)
I.	a	b	— —
II.	—	b	A —
III.	a	—	— B
IV.	—	—	A B

Aside from the support offered by Koeckert, in his demonstration that specific absorption experiments prove the presence of these bodies, further confirmatory evidence is found in the fact that the agglutinogenic character of cells is demonstrable in the early months of life, whereas the agglutinins do not appear until near the end of the first year. It is also stated that transfusion with a certain group may lead to the development in the recipient of specific iso-agglutinins for the group injected. Kolmer's work, however, shows that immunization of animals with the blood of the various groups produces a hemagglutinative and hemolytic serum without group characters. Karsner and Koeckert have shown that desiccation leads to a loss of specificity of the sera, and that at a certain period in the desiccation a common agglutinin is found which clumps the cells of all groups, including Group I. This is probably in part due to alterations in physical character of the redissolved sera, and to alterations in hydrogen ion concentration, as shown by Karsner and Collins. Therefore, although the Landsteiner hypothesis offers an excellent working basis, it seems probable that an intricate physico-chemical mechanism is largely concerned in the phenomenon of iso-hemagglutination.

Iso-hemagglutinins in Lower Animals.—The presence of iso-hemagglutinins in animals other than man is extremely irregular and infrequent. Certainly no classification into definite groups has so far been demonstrated. In our own experience the examination of from ten to twenty members each of dog, rabbit, cat, and guinea-pig species has failed to show iso-hemagglutinins, but others who have examined larger numbers have found an occasional instance of iso-hemagglutination.

Relation of Iso-hemagglutinins to Blood Transfusion.—The principal importance of iso-hemagglutinins and the related iso-hemolysins in human medicine relates to the transfusion of blood, a therapeutic measure which civil and military practice have shown to be of the utmost value in combating secondary anemia following hemorrhage. It is also recommended for prolonged sepsis with or without severe anemia, for primary anemias, and for certain other

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diseases, but results are not so brilliantly successful as in secondary anemias, particularly those resulting from acute hemorrhage. Ill effects following transfusion are spoken of as reactions and include fever, chills, cyanosis, hemoglobinuria, and even death. Cases coming to autopsy show parenchymatous degenerations of solid organs, marked congestion of all viscera, acute splenic hyperplasia, hemoglobin staining, and sometimes multiple small emboli of agglutinated erythrocytes. Blood studied in life has shown phagocytosis of erythrocytes by the recipient's white corpuscles. The reactions depend in large part on intravascular agglutination and hemolysis, but probably certain other factors play a part. The prevention of these other factors awaits the determination of their nature, but the avoidance of agglutination and hemolysis can easily be accomplished by use of the very simple tests for the determination of the presence of conflicting iso-agglutinins. The simplest of these tests is the determination of the groups to which recipient and prospective donors belong. The most desirable means of selection, in our opinion, is that whereby the donor is chosen from the same group as the patient. Lee and others have maintained that it is equally safe to use members of Group I as donors for recipients of any group. The argument in favor of this procedure is based on the statement that the real danger in transfusion is the use of a donor whose cells are agglutinated by the recipient's plasma and that the converse has little or no significance. The cells of Group I are not agglutinated by any sera and are, therefore, safe to use. In our own experience we have seen occasional reactions following this procedure and prefer to use a donor in the same group as the recipient. Reactions following the general use of Group I donors do not necessarily mean that the trouble is the result of agglutination or hemolysis, for, as has been indicated above, other factors may be concerned. Nevertheless, it holds true that thousands of transfusions have been done with Group I as the "universal" donor and without reaction. The explanation of the fact that a donor may thus be used, whose plasma or serum is capable of agglutinating the recipient's erythrocytes *in vitro*, is not settled, but certain theories have been offered. It must be remembered that in transfusion a small bulk of blood is introduced, as compared with the total bulk in the recipient's body. Therefore, agglutinins introduced in this way are much diluted, and as they ordinarily occur in low titer they may be sufficiently diluted to be ineffective. Another possibility is that the agglutinins are absorbed equally by an extremely large number of cells, each cell, therefore, taking up too small an amount to be subjected to agglutination. A third possibility is that an excess of non-agglutinable cells and the presence of the patient's own plasma permits of the formation of only small clumps of cells, so small that they are of no significance in the circulation. Our own work has failed to demonstrate anti-agglutinins in a large number of tests, and it seems improbable that a mechanism of this type operates to protect the recipient. It is conceivable, however, that these possible factors of safety may not operate and

reaction follow this type of transfusion. We cannot enter here into a discussion of methods of transfusion.

Methods for Testing Human Blood.—The simplest method depends upon the preservation in the laboratory of known Group II and Group III sera. These should be selected so that they have a relatively high titer, and should not be employed if they titrate less than 1 to 16. The method to be described is essentially that of Lee and Minot. The apparatus includes a few 7x75 mm. test-tubes, a platinum loop, microscope slides with at least one built up on the ends with pieces of glass rod or match sticks glued on by means of balsam so that another slide may be inverted upon it with hanging drops. A microscope is useful but not essential, since a hand lens of 10 diameters magnification is satisfactory. A small moist chamber is desirable but not essential. In well equipped laboratories the serum may be kept in the ice chest in sterile ampoules or small bottles and drops removed as required. Somewhat more satisfactory is preservation in sections of drawn out glass tube similar to that used for vaccine virus. Each small tube contains serum for one test and the serum may be blown out exactly as is done with vaccine virus. Phenol 0.5 per cent. may be used as a preservative. One-half cubic centimeter of physiological salt solution is placed in a test-tube, and to this are added one or two drops of blood, obtained by ear or finger puncture, sufficient to make a slightly opaque emulsion. Clotting of the mixture is not harmful since subsequent shaking of the tube will produce a homogeneous suspension. Upon a microscope slide are placed one drop each of the sera of Groups II and III. With the platinum loop a drop of blood suspension is mixed, by gentle rubbing, in each of the serum drops and the slide immediately inverted upon the prepared slide or a small rack so as to make hanging drops. At the end of five or ten minutes the reaction occurs and may be seen with the naked eye; in order to avoid mistakes owing to slight agglutination it is important to observe with the 16 mm. lens of the microscope or a hand lens. If a small number of specimens is examined it is well to have controls with known I, II or III cells. If the reaction is delayed the slide should be kept in a moist chamber for one-half hour and then observed. The group to which the cells belong is determined by the following section from the chart of inter-agglutination:

		STANDARD SERA	
		II.	III.
Cells	I.	—	—
	II.	—	+
	III.	+	—
	IV.	+	+

Thus if the cells are agglutinated by both sera they belong to Group IV; if not agglutinated at all and the control cells show that the sera agglutinate properly the cells belong to Group I; if agglutinated by only III serum they belong to Group II, and if agglutinated by only II serum they belong to Group III.

Hanging drops are not essential, but serve to make the reaction somewhat clearer. The reaction occurs with the slides upright. In this case cover slips may be used. Many employ undiluted blood and cover with cover slips, but rouleaux formation sometimes offers a confusing picture.

It has been suggested that since the important point of determination is as to whether or not the donor's corpuscles are agglutinated by the patient's serum, the latter may be separated and placed on a slide with the donor's corpuscles. The separation of the serum requires more time than a complete test as given above, and is subject to serious error if the patient's serum happens to be of low agglutinin titer.

If standard sera are not available they may be prepared if a known II or III blood can be obtained. The interaction of the cells and serum with fifteen or twenty other bloods can be worked out on the basis of the chart on inter-agglutination. Space does not permit of giving the details, but Brem's method gives them accurately. If this cannot be done the method of Rous and Turner is probably the best of the methods for use where standard sera are not to be had, since this method determines the activity of both the cells and serum of the donor and recipient. The method with slight omissions is taken directly from the article of Rous and Turner in volume 64 of the Journal of the American Medical Association.

"Collection of the Blood.—The blood is taken from the patient and the prospective donors in a 1-10 mixing pipette, such as is used in counting leucocytes. The pipette is rinsed beforehand with 10 per cent. sodium citrate in water; the citrate solution is drawn up to the mark 1; the pipette is rapidly filled with blood from a puncture of the ear or finger; and without pause the mixture is expelled into a small, narrow test-tube. There is thus obtained a citrated blood containing slightly less than 1 per cent. of citrate. The pipettes which we have employed hold only 0.25 c.c. of fluid. This much blood is easily obtained from a single puncture. There is no objection to increasing the flow by pressure. Should it cease before the pipette is full, the blood must be at once expelled into a test-tube, in order that it may mix with the citrate and clotting be avoided. The mixture is then taken up again, a new puncture made, and the pipette completely filled. After each blood is obtained, the pipette is rinsed with citrate, then with distilled water, then with fresh citrate, and it is ready for another blood. If several donors are to be tested, two pipettefuls of citrated blood should be obtained from the patient. It is best to take them from different puncture wounds, in order to avoid a possible clotting in the pipette.

"Mixing.—The mixing is done in pipettes with a capillary end—the so-called Wright pipettes obtained by drawing out glass tubing in the flame. (Fig. 11.) The citrated bloods are used as such, and two combinations are made of the patient's blood with that of each prospective donor, a mixture containing nine parts of the patient's blood to one of the donor's, and a mixture of equal parts of the two. The proportions used need be only approximate. In case of emergency the first of the mixtures will suffice, since by its use the most dangerous possibility, namely, that the blood of the recipient might destroy that of the donor, can be ruled out. Following the technic usual with Wright pipettes, the capillary tube is marked, blood is drawn to the mark, and each column of the blood is separated by an air bubble from the next that is drawn up. To insure proper mingling, each mixture should be expelled on a slide, or Widal plate, and then drawn high in the pipette, which may be sealed off in the flame in case the examination is not to be made for some time.

"Incubation.—No incubation in the ordinary sense is necessary. The pipettes are kept at room temperature, and readings are begun after two minutes if there is need to hurry. Readings are for agglutination, and even within two minutes this is plainly evident, except when the agglutinating forces are notably weak. In the final choice of a donor it is safest to rely on results obtained after the mixtures have stood for fifteen minutes. But the ruling out of individuals with unfit blood may be begun practically at once.

"Readings.—The capillary end of each pipette is broken, a small drop of the blood expressed on a slide, a large drop of normal salt solution superimposed without mixing, a coverslip put on, and the preparation examined for agglutination under the microscope. Fresh preparations can be made at intervals if desired. The salt solution is not absolutely necessary; but very clear pictures are obtained as the blood spreads in it. When agglutination has occurred, the red cells show a characteristic clumping, sometimes in small masses, often in large ones that are very evident microscopically.

"If there is no clumping in the preparations made after the mixtures have stood fifteen minutes, the assumption is warranted that the bloods do not agglutinate or hemolyze each other. But if clumping is present in the 9-1 mixture and to a less degree or not at all in the 1-1 mixture, it is certain that the blood of the patient agglutinates that of the donor, and may perhaps hemolyze it. Transfusions in such cases are dangerous. Clumping in the 1-1 mixture with little or none in the 9-1 indicates that the plasma of the prospective donor agglutinates the cells of the prospective recipient. For practical purposes these findings suffice. But if there is a desire to know whether both bloods contain agglutinins, a 1-9 mixture should be made. If this and the 9-1 mixture show large clumps, whereas the clumps are smaller when the bloods are mixed in equal parts, two agglutinins must be present. Should there be only one agglutinin, little clumping or none will be observed when the blood containing the agglutinin is diluted with nine parts of the other blood."

By the use of the technic indicated in the last paragraph, it is possible to overcome error due to weak agglutinin content of the recipient's blood. This we believe is of especial importance if the patient has been ill for a long time.

Reactions to Transfusion.—The effects on the body of introducing high titer hemagglutinins have been studied experimentally in normal animals and in those which have been splenectomized. In normal animals there is found agglutination of red blood-corpuscles with embolism in liver, lungs and other viscera. The liver is often found to show hyaline necrosis in connection with the emboli. The spleen is large and diffuent, and there are small areas of necrosis as well as phagocytosis of erythrocytes by endothelial cells. Necrosis is also found in the follicles of lymph-nodes. Multiple hemorrhages may also be noted. After splenectomy the phagocytic function of the spleen is taken over by the lymph-nodes. The incident hemolysis in either case leads to hemoglobinemia and hemoglobinuria, but in splenectomized animals the threshold of excretion of hemoglobin is somewhat higher than in normal animals.

In severe and fatal reactions in man the phenomena are not likely to be so marked. Phagocytosis of erythrocytes by the recipient's leucocytes has been observed. We have performed autopsies on twelve cases in which transfusion was practised shortly before death, in three of which the death was at least in part due to the use of unsuitable blood. In all of these the spleen was considerably enlarged (170, 390, 400 grams), and in one there were multiple small hemorrhagic infarcts. One case showed enlarged soft white lymph-nodes. The bone marrow was normal in all. All showed marked cloudy swelling of the kidneys. Two showed hemoglobinuria, and in one of these there was post-mortem staining by hemoglobin. In the case with multiple infarcts of the spleen 50 c.c. Group II blood had been given to a Group III recipient, and there was neither hemoglobinemia nor hemoglobinuria. In the case with hemoglobinemia and hemoglobinuria about 700 c.c. Group III blood was given a Group I recipient. Unfortunate accidents led to these errors, and the groups were discovered subsequent to the operations. In the nine cases where the transfusions were satisfactory the spleen was either normal or if enlarged was accompanied by septicemia.

Chemical Agglutination of Erythrocytes.—Blood-corpuscles are agglutinated not only by various sera but also by certain chemical substances. Gay has examined the function of the tonicity of the surrounding medium in determining iso-hemagglutination and maintains that the bloods of that group whose cells are non-agglutinable (Group I) are constantly of higher total molecular concentration than the other bloods. He further states that a "simple hypertonic solution of CaCl_2 , but more particularly solutions hypertonic both in respect to NaCl and CaCl_2 , produces a cohesion of any human blood after several hours resembling iso-agglutination." Studies of hypotonic solutions and of variations of any considerable degree in hydrogen ion concentration have been rendered difficult because hemolysis is likely to occur under these conditions and render conclusions difficult. Landsteiner and Jagic in 1904 were the first to call attention to the fact that a well-defined colloid, namely silicic acid, agglutinates erythrocytes.

Gengou reported agglutination and hemolysis by means of such chemical precipitates as calcium fluoride and barium sulphate, but in these instances serum served to prevent agglutination. This appears to be another example of protective colloidal action. According to Girard, Mangin and Henri, the red cells carry electro-negative charges, but agglutination has been produced by colloids regardless of the electrical charge they carry. We, in collaboration with Hanzlik, have examined a wide variety of colloids and have determined that many of those which produce thrombosis upon intravenous injection into animals also produce agglutination in the test tube.

Conglutination.—Bordet and Gay, as well as Muir and Browning, independently described in 1908 the phenomenon of congutination—an agglomeration of corpuscles in the presence of two normal sera. The result of this reaction is the agglutination of corpuscles, but what is known of its mechanism makes it advisable to consider the phenomenon after the discussion of hemolysis (see page 126).

PRECIPITATION

Introduction.—The discovery of agglutination led to the discovery by R. Kraus in 1897 of the precipitin reaction. His problem was to determine whether or not agglutinating sera would act in any way on extracts of bacteria, and in his work with typhoid bacilli and cholera vibrios he found that the addition of the specific antisera to the bacterial extracts led to the formation of a precipitate and that this reaction is specific. This was confirmed by Nicolle. Previously Widall, Levy and Bruns had shown the converse, namely, that filtrates of typhoid and cholera cultures upon injection led to the formation of agglutinins. In 1899 Tchistovitchs published the results of his work with horse serum and eel serum, demonstrating the formation of specific precipitates when the serum of rabbits previously inoculated with these sera was added to the antigenic sera. Bordet confirmed this with chicken serum and later showed that cow's milk upon injection induces the formation of a specific precipitating serum for the casein of the milk. Kraus states that previous to the publications of Tchistovitchs and of Bordet he had also, in collaboration with Winterberg and E. P. Pick, experimented with proteins of animal origin. Fish demonstrated the specificity of various milk antisera for their respective antigens. The reaction was enlarged in scope for various other animal proteins. Kowarski showed that the reaction is specific for higher vegetable proteins as well as for those of bacteria. Certain authors have claimed that peptones, globulins, albumoses and other protein products are antigenic in a similar manner, but the weight of evidence is that the whole protein molecule is necessary. A recent review of the literature on this subject by Fiñk has shown that statements in regard to the proportion of the entire protein molecule necessary to take part in the reaction are confusing and obscure. Frequently, instead of testing against the decomposition product itself, the serum obtained by its use has been tested against the entire protein molecule.

Fink worked with the precipitates obtained by salting protein solutions and found that rabbits inoculated with one-fourth, one-third, one-half, and two-thirds saturation products produced no precipitins nor complement-fixing bodies. In guinea-pigs, however, the three-fourths saturated and completely saturated products possess slight sensitizing and intoxicating properties, the latter being apparently the more active. Nevertheless, three-fourths saturated and completely saturated products of egg-white were sufficient to produce definite formation of precipitin and complement-binding antibodies but not in as high a titer as entire protein.

Nature of the Reaction.—In analogy with the terms used in the phenomenon of agglutination Kraus named the antigen, precipitinogen and the immune body precipitin. The reaction is similar to agglutination in all respects save that here we have to deal with proteins in solution. Aging or heating leads to the formation of precipitoids, group reactions as well as inhibition zones appear, heat has much the same influence in all respects as in agglutination, salts play an important part in the reaction and specific absorption can be demonstrated. It is known, however, that some protein molecules are largely built up of alkaline amino-acids and that others are built up largely of the acid amino-acids. Salmine, for example, a product of the spermatozoa of certain fish, consists almost entirely of strongly alkaline amino-acids. Gliadine of wheat is chiefly built up of dibasic amino-acids, glutaminic acid. The fermentation end product of salmine is alkaline and of gliadine acid in nature. An antigliadine serum gives with a homologous precipitinogen, a beautiful precipitate, while a mixture of salmine and antisalmine-serum gives no visible precipitate. This would indicate that the alkaline salts are of importance in the actual formation of the precipitins, and we know by simple titration that during the precipitin reaction there occurs a reduction of acidity. Nevertheless, it is also asserted that when the acidity is due to an organic acid or acid salt the reaction appears to be promoted. The precipitin is precipitated in the euglobulin fraction of the serum, is destroyed slowly by trypsin and rapidly by pepsin. The immune serum contains the precipitin which constitutes the bulk of the precipitate, the latter thus representing, according to Wells, "the insoluble modification of the previously dissolved precipitin and originates chiefly or entirely in the proteins of the immune serum." Welch and Chapman obtained, with a precipitinogen containing only 1 gram of protein, a precipitate containing 21.1 grams of protein. Pick employed a precipitinogen which did not give the biuret reaction and with this obtained a voluminous albuminous precipitate. It must not be understood that precipitins are always the result of immunization, for Vaughan states that goat serum contains a normal precipitin for rabbit and for dog sera. Such normal precipitins are not of very high titer and are not so sharply specific as the immune precipitin. Puppies, kittens and rabbits up to ten days old may absorb native protein from the milk of the mother which apparently stimulates the formation of precipitins. Sera of human

infants have been observed to precipitate the protein of cow milk. It appears possible, then, that from absorption through the intestinal tract early in life the protein may appear in the circulating fluid in native form and thus stimulate the formation of precipitins. These, of course, are not normal precipitins in the sense indicated above for goat serum but similarly are always precipitins of low titer and not highly specific.

Experimental Demonstration.—For practical demonstration of the reaction the serum proteins are the simplest to use. For immunization of animals the intravenous route is by far the best, injecting 2.0 c.c. serum at five-day intervals and bleeding ten days after the last dose. Three doses are usually sufficient, but five doses frequently give a precipitin of very high titer. In order to get clear serum it is necessary to fast the animal for twenty-four hours before bleeding, thus eliminating fat from the serum. Rabbits are the animals usually selected for this purpose because of their availability in the laboratory and because of the relative ease of intravenous injection. Hektoen has shown, however, that the domestic fowl is a prompt, reliable and liberal producer of precipitins, even more so than the rabbit. A single intraperitoneal injection of 20 c.c. of defibrinated blood or serum in most cases yields at the end of ten or twelve days a precipitating serum of sufficient strength and specificity for practical purposes; but on account of an unwelcome tendency to give non-specific reaction, great care must be exercised in all the tests with fowl antiserum, and it is necessary to use salt solution 1.8 per cent. in strength. Man is also a good producer of precipitins, as has been shown by investigation of human serum after the individual has been given doses of horse serum. For performing the test, narrow tubes, not more than 5 mm. in diameter are most suitable in order to save reagents and get clear-cut results. Instead of diluting the antiserum, it is customary here to dilute the antigenic serum. Nevertheless the titer thus obtained is referred to the immune precipitin. Two methods are in use, the original method of actual precipitation, and the Fornet ring test. In either case dilutions of the antigenic serum are made 1-10, 1-100, 1-1,000, 1-10,000, 1-100,000, and 1-1,000,000, with provision for a salt solution control. After such a preliminary test the serum may be more accurately titrated with intermediate dilutions. For determining precipitation 1.0 c.c. of each dilution is run into tubes with a nipple pipette, and to each is added 0.1 c.c. immune serum, the latter settling into the dilutions, without shaking. Immediate observations are made and then the mixtures incubated for one hour at 37° C., followed by subsequent observation, and if desirable further observation after twenty-four hours in the ice chest. The Fornet ring test is more clear-cut and is more commonly used. Here 0.1 c.c. immune serum is placed in the tubes and the dilutions of antigen added with nipple pipettes, so as to form a contact ring as in the Heller test for albuminuria. A white ring gradually spreading both up and down indicates a positive reaction. A good immune serum titrates 1-10,000 or more, although titers of 1-100,000 are obtainable.

The production of bacterial precipitins is somewhat more difficult and requires longer immunization, the precipitins appearing, as a rule, somewhat later than the agglutinins. Zinsser recommends the use of salt solution emulsions of agar cultures killed at 60°-70° C., rather than extracts or filtrates of broth cultures. The intravenous route is best unless the bacteria are extremely toxic, when the subcutaneous or intraperitoneal method may serve. Intravenous injections should be given four or five times at five- or six-day intervals, the animal (rabbit) being bled eight or nine days after the last injection. Extracts of bacteria for similar purposes are obtained by growth for three weeks to three months in slightly alkaline broth, filtration through Berkefeld filters and injection of the filtrate. Salt solution suspensions of agar cultures may be shaken in a machine for twenty-four

hours, filtered through a Berkefeld filter and the filtrate used. Kraus, in his original studies, used broth filtrates and also juice expressed from the bacteria. Kraus points out that the broth filtrates of toxin-producing organisms such as *Bacillus diphtheriae* do not precipitate when mixed with antitoxic serum. That this is a general rule, however, is not true, since Jacoby has shown that it is possible to obtain a precipitate by mixing ricin and antiricin serum, and others have observed similar reaction with the use of abrin and antiabrin serum as well as croton and anticroton serum.

The delicacy of the precipitin reaction is great and only exceeded, in certain respects, by complement fixation and the anaphylaxis reaction. It is of interest to note that whereas the Biuret and the Millon test for protein will hardly exceed dilutions of 1-1000, the precipitin reaction will detect not only the presence of protein but the species from which it originates, commonly in dilutions of 1-10,000 or 1-20,000 and even 1-100,000.

Physical Basis of Precipitation.—The influence of heat on precipitation and also the group reactions are of considerable importance in the practical application of the phenomenon and will be dealt with more fully as this side of the question is considered. The comparisons offered between agglutination and certain colloidal phenomena (see page 94) are equally applicable to precipitation and require no extensive discussion here. It must be borne in mind, however, that the colloidal interpretation of these phenomena is not proven. Essentially the same arguments are available against the conception of precipitoids as against that of agglutinoids, but none of these explains satisfactorily the specific absorptive capacities of these hypothetical bodies. As agglutinogen and agglutinin may exist in the blood of a living animal, so may precipitinogen and precipitin coexist. This is compared by Zinsser to the fact that if gum arabic is added to a mixture of thin gelatin and arsenic trisulphide the precipitation which ordinarily occurs will be prevented. The gum arabic in this instance is a protective colloid. It is assumed that such a protective colloidal action operates to prevent precipitation when precipitinogen and precipitin coexist in the blood of a living animal. After the blood is withdrawn and allowed to stand, this protective action disappears and precipitation occurs.

The fact that precipitin and precipitinogen can coexist in circulating blood and that experiments on the attempted production of iso-agglutinins with their conflicting results has led to the question of whether or not it is possible to produce precipitins in an animal by the injection of proteins of a closely related species. Uhlenhuth and Weidanz claim to have produced precipitins for human serum by injecting human serum into monkeys, the resulting precipitin acting on human but not on monkey serum. Berkeley and later Sutherland were unable to confirm this experiment and we are forced to the conclusion that precipitin formation in closely related species is by no means a constant phenomenon. Such precipitins would be practically

iso-precipitins, and, as we have seen, their existence is irregular and questionable.

Practical Application.—Wladimiroff first applied precipitation practically in the diagnosis of glanders in horses, using the serum of suspected horses against a filtrate from cultures of glanders bacilli. Kraus employed the reaction to identify closely-related bacteria. At the present time, however, agglutination is employed for the detection of glanders and also for identification of bacteria rather than precipitation, because the latter procedure introduces the more cumbersome technic of obtaining filtrates.

The Forensic Blood Test.—Uhlenhuth and Beumer published their first results on the use of the precipitin reaction in legal medicine in 1903. Other studies were rapidly contributed, and to-day the method has an established place in the identification of stains by blood and other fluids such as seminal fluid. If spots on clothing or other material are suspected of being blood, this must be proven by chemical, microscopic or spectroscopic examination. Subsequently the precipitin test is used to determine the species from which the blood originated. Before proceeding to this test it is necessary to have immune precipitating serum against the suspected species, usually man, an additional immune precipitating serum against some other species and a normal rabbit serum. The immune sera are prepared according to the method outlined on page 108. The suspected material must be carefully guarded against possible substitution or contamination until the immune sera are prepared. It is then dissolved in physiological salt solution and a perfectly clear filtrate used. If the material is on cloth the latter should be teased so as to permit of solution; if on some solid material, such as a knife blade, it should be scraped off, ground in a mortar and a small amount of salt solution added. Cloth should be placed in a test-tube or bottle, and it is well to have a control with unstained cloth. The time for extraction depends to a certain extent on the freshness of the material, but it is wise to allow it to extract in the refrigerator over night, adding a few drops of chloroform to prevent bacterial growth. If extraction does not proceed well in salt solution it may be necessary to extract with 1 per cent. potassium cyanide solution, correcting the alkalinity after extraction, by means of tartaric acid. To prove that the solution contains protein a small amount may be boiled and treated with acetic or nitric acid as in the ordinary test for albuminuria. A final solution of the suspected material in a dilution of 1-1000 is usually employed, and this dilution may be approximately determined by the foam test. For this purpose make a 1-1000 solution of any convenient serum, blow air through it in a test-tube and note the persistence of bubbles above the fluid. Dilute the extract gradually and blow air through it, repeating until that dilution is obtained which will produce a foam of about the same viscosity as that in the control tube. If the solution is not perfectly clear it may be centrifuged or passed through a filter of washed asbestos or cotton. On the assumption that the spot is suspected of being human blood the test is set up as follows:

Tube	Material	Result
1	Suspected extract + anti-human serum	+
2	Suspected extract + normal rabbit serum	—
3	Control extract + anti-human serum	—
4	NaCl solution + anti-human serum	—
5	Human serum (1-1000) + anti-human serum	+
6	Beef serum (1-1000) + anti-human serum	—
7	Sheep serum (1-1000) + anti-human serum	—

The amounts throughout are 0.1 c.c. of each reagent, and the test is made by the Fornet ring method. Inasmuch as acceptable antisera should titrate 1-10,000, the reaction in 1-1000 dilution occurs within a few minutes, and the above result would be interpreted as a clear-cut positive for human blood provided the preceding chemical and other tests for blood had been positive. It should be remembered that the precipitin reaction in this instance simply identifies the material as human protein, and a similar result might be obtained from human seminal fluid, albuminous urine, purulent sputum, exudates and transudates, unless the preliminary tests had been carried out.

Biological Relationships.—The question of the specificity of this reaction has been somewhat confused by quotation from the famous studies of Nuttall in regard to interrelationship of species. Nuttall's book, published in 1904, was of the utmost importance to biology in general, because it demonstrated anew by the use of the precipitin reaction the interrelationship of animal species. He showed, for example, the close biological relationships between man and the higher ape, also similar relationships in the lower animals, as between the goat and the sheep, the horse and the ass. Reference to the tables which he published would seem to indicate, however, that the relationship between man and the higher ape was so close as to be indistinguishable by the precipitin reaction. An example in point is the statement which he makes that whereas human blood will respond to antihuman precipitating serum to the extent of 100 per cent., the blood of the chimpanzee responds to the extent of 130 per cent. These figures, however, refer to the bulk of the precipitate thrown down in relation to standard dilution of the various bloods employed. He used relatively low dilutions, allowed the sera to remain in contact for several hours and then measured the amount of precipitate. This, as can readily be seen, is different from the method which is employed at the present time in determining the titer of the sera against the immune serum. The latter method is distinctly more delicate in determining the specificity of the reaction. For that reason it is the method employed in the forensic test of to-day, as well as in ordinary laboratory procedures. Furthermore, we find at the present time that the test demonstrates its specificity particularly in the presence of strong sera by reading very shortly after the contact has been made. Hektoen offers an excellent example of this in the following table (antihuman serum) :

Blood	
Fish	1-10
Chicken	1-10
Rabbit	0
Guinea-pig	1-10
Rat	1-10
Cat	1-10
Dog	1-10
Swine	1-10
Sheep	1-10
Beef	1-10
Horse	1-10
Goat	1-10
Monkey (<i>Macacus rhesus</i>)	1-100
Human	1-5000

It will be noticed by reference to the above table that the titer of the serum used in this particular test was only 1-5000, and we would expect an even greater difference between the titer with the different animal sera if the antihuman serum had been of higher titer. Concerning the group reactions in the precipitin test an interesting instance is given by Hamburger in regard to the action of the serum of a rabbit inoculated simultaneously with sheep serum, goat serum and ox serum, all of which are fairly closely related to each other biologically.

The serum of the rabbit when mixed separately with each of these three antigenic sera gave the most voluminous precipitates in the presence of the sheep serum, less in the presence of the goat serum and least in the presence of the ox serum. This observation has been confirmed by Arrhenius. Just why the sheep antiserum should be the most powerful is difficult to say, but it might be assumed that the sera of closely-related species may augment the antigenic power of the strongest of the three species used. Of further interest, Hektoen has shown that in rabbits previously injected with foreign serum the subsequent injection of a different serum may reawaken the production of precipitin for the antigen previously injected. The practical value of this fact is that rabbits which have once been used for the production of precipitin should not be used again for the same purpose with another protein because of possible decrease in specificity of the second antiserum.

Organ Specificity.—The question of organ specificity is of considerable importance in the discussion of the specificity of the precipitin test. Numerous experiments have been made by various immunological methods to determine whether or not it is possible to identify the protein of a given organ within the same species. It may be stated very briefly that these experiments have not met with any great degree of success. However, in regard to the protein of the crystalline lens of the eye and the protein of the testicle, certain interesting facts have been discovered. Immunization with protein extracts of the crystalline lens will produce precipitating sera which operate not only on the lens protein of the same species but on the lens protein of all animals as low in order as fish. In this example the species specificity has been entirely replaced by a curious organ specificity. The organ specificity in this case is so strict that the immune serum will not react with other tissue extract even of the same species. Lens protein may, indeed, be injected into the same species from which the lens was taken and give rise to specific precipitins. By the use of the complement-fixation test it has further been shown that in adult human beings it is possible to detect the presence of an antibody for lens protein which is not detectable in children. This phenomenon will be mentioned later in connection with the autocyctotoxins (see page 142). Zinsser comments to the effect that biologically these phenomena probably signify that although there are fundamental species differences between the general body proteins of various animals, there are still in certain highly specialized organs varieties of protein which possibly because of functional exigencies have developed similar chemical characteristics. In regard to the testicle and the placenta, it might be supposed that the germ character of these tissues is retained as distinctive from the somatic character of the other body tissues. This would not apply to crystalline lens, since it is not of germ character. On the other hand, although the lens can be regarded as a highly-specialized organ in both morphological and physiological senses, the testicle and the placenta can hardly be so considered. Such discussions are likely to be fruitless

until it is possible to isolate the protein of other body organs without contamination by the animal's blood. Up to the present time this seems to be impossible. Studies by Bell, for example, with perfusion of various organs has demonstrated the impossibility of removing the blood completely.

Detection of Food Adulteration.—The precipitin reaction is applied not only to detect blood as indicated above but also various other body proteins; for example, it may be used to detect the nature of bone fragments or other tissue scraps. Of great significance is the fact that the precipitin test is employed for the detection of adulteration of food products. It has been utilized, for example, in detecting adulteration of sausages by the use of horse and other meats. In the preparation of such food products, heat is often employed, and therefore it is necessary to know the influence of heat on the precipitin reaction. The relation of heat to the agglutinin reaction has already been discussed (see page 93), and it is found that similar conditions exist in regard to the precipitin test. Obermeier and Pick studied this problem experimentally and found that an antiserum, even of high titer, produced by an unheated antigen, failed to precipitate when brought into contact with heated serum. If, however, animals are immunized with serum boiled for a short time, the resulting immune serum forms a precipitate when brought in contact with either heated serum or unheated serum. Therefore, the precipitin produced by the latter method is regarded as more comprehensive in its precipitating activity, but nevertheless its species specificity remains unimpaired. By employing a lower degree of heat, namely 70° C., Schmidt found that this marked difference was not so apparent and that an immune serum prepared by injecting unheated serum would produce precipitation with unheated serum and with the moderately-heated serum. However, the titer of antiserum prepared by the use of moderately-heated antigen was not as high as with the use of unheated antigen. Schmidt further found that he could produce an even more comprehensive immune serum by boiling the antigen until a coagulum was formed, namely, for three hours. The coagulum was washed with salt solution, dried, powdered and then taken up with a normal NaOH solution. Zinsser and Ottenberg found that the use of a boiled antigen led to the production of a comprehensive precipitin, but nevertheless they determined that this resulted in some loss of specificity of the precipitin.

This outline of the influence of heat will serve to show that in the detection of the adulteration of food products extreme care must be taken in the selection of material. Wherever possible, fresh material should be obtained, and the material for testing should always be taken from near the middle of the specimen. This precaution prevents contamination with other meat, and in the case of sausage yields material likely to be less influenced by heat or smoke. The meat is cut into fine pieces and allowed to extract in salt solution. Clarke used 30 grams meat and 50 c.c. physiological saline, extracting in the ice chest for twenty-four hours, and further diluting 1-300 for the test. Such

extracts must be proven as to protein content by the nitric acid test and the foam test. Violent shaking is to be avoided, because it liberates fats and lipoids which cloud the extract. If precipitation occurs by the use of this extract with an immune serum prepared by injecting unheated protein, the test can be regarded as highly specific. If, however, it is necessary to use serum which is prepared by injecting heated protein, the specificity cannot be regarded as being so high. In practice it is the rule to use serum prepared by injecting unheated protein rather than otherwise, unless the special indications of the case indicate the use of an immune serum prepared from heated antigen. The technic in case of food adulteration is essentially the same as for the detection of blood. Inasmuch as the specificity of this reaction is a species specificity, it is satisfactory to utilize the animal's serum for immunization rather than extracts of the flesh under suspicion.

In mixtures of meat such as one finds in sausages, the mixture in itself sometimes interferes with the delicacy of the test. In these cases it has been found that the complement-fixation test is likely to give more satisfactory results.

The precipitin test is also applied in the enforcement of game laws. For example, cases arise in which the unlawful possession of venison is suspected, and the identity of the meat may be established by the precipitin reaction.

Numerous suggestions have been made regarding the identification of racial strains within species, but we agree with Hektoen in saying that "suggestion to the contrary notwithstanding, it is not possible to distinguish between different human races, and far less between individuals, by means of the precipitin test."

Function of Precipitation in Immunity.—The function of precipitation in the protection against infection is not clear, and, indeed, according to certain theories, it may play a part in hypersusceptibility. Friedberger has shown that the addition of complement to a precipitin-precipitinogen mixture leads to the formation of a toxic body, but there is no convincing evidence that this actually takes place in the living animal (see page 218). It is to be considered possible, on the other hand, that a certain amount of protection against foreign proteins may depend on precipitation, the precipitate being less harmful and more susceptible to the destructive action of ferments.

CHAPTER VI

CYTOLYSINS

INTRODUCTION.

HEMOLYSINS.

IMMUNE HETERO-HEMOLYSINS.

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QUANTITATIVE RELATIONS OF AMBOCEPTOR AND COMPLEMENT.

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SUMMARY.

Introduction.—In the study of resistance to disease it was learned very early in the course of the investigations that the blood serum possesses the property of destroying bacteria. Later it was found that blood serum may possess similar power in regard to other cells, including various animal cells, particularly the erythrocytes. Rather than consider the subject of cytolysis in a historical fashion, we believe that it may be much more clearly discussed by first presenting the established facts which have been learned concerning the power of blood serum to destroy red blood-corpuscles. Many substances other than blood serum may destroy erythrocytes and immunology has profited from the study of hemolysis resulting from chemical and physical agents, but the greatest advance has been made by the investigation of the hemolytic properties of the blood.

Hemolysins.—Hemolysins are classified, in the same manner as hemagglutinins, into autohemolysins, iso-hemolysins and heterohemolysins. These may be present normally or may be produced as a result of immunization. Landois in 1875 studied those normal heterohemolysins which for years have made blood transfusion a dangerous operation and showed that fresh sera from various species possess the power of dissolving or laking the erythrocytes of certain foreign species. In 1898 Belfanti and Carbone noticed that the serum of a horse which had received numerous injections of rabbit blood was, upon injection, specifically toxic for rabbits, but they did not determine the cause of the toxicity. In the same year Bordet published his discovery of the fact that several injections of defibrinated rabbit blood into the peritoneal cavity of guinea-pigs led to the production of an immune body in the guinea-pig serum capable of rapidly laking rabbit erythrocytes, whereas normal guinea-pig serum possesses the same property in only slight degree or not at all. Shortly afterward von Dungern and Landsteiner independently published similar results. The immune body in the serum has been named hemolysin and also hemotoxin, but the former term has received much wider usage, because the constitution of this body is not that of true toxins, because the effect is seen on the blood-corpuscles rather than the whole blood, and because the hemoglobin is liberated for solution in the surrounding medium without actual destruction of the stroma. Bordet in his study of the subject showed that heating the serum to 55° C. for thirty minutes so altered it that it no longer produced hemolysis; in other words, it became "inactive." It could, however, be reactivated by the addition of a small amount of fresh normal serum. This indicated that two substances are concerned in the hemolytic activity of blood serum, a thermostable substance present in the immune blood and a thermolabile substance present in normal blood as well as in immune blood. Bordet named the immune thermostable body "substance sensibilisatrice" and Buchner named the thermolabile body "alexine." Ehrlich and Morgenroth, whose studies have been of fundamental importance, named the thermostable body "amboceptor" and the thermolabile body "complement." Others have given other names, but these two forms of

nomenclature are most frequent in the literature. We have elected to use the terms amboceptor and complement because of our belief that these terms have attained the more widespread usage. Complement appears in the blood of many species, but may be very small in amount or absent from certain species. Certain complements may operate with the amboceptors of only a few species, whereas others may act with amboceptors of a large number of species. Within a given species different individuals may possess complement in variable quantity, and it may vary at different times in the same individual. The complement of guinea-pig blood is usually large in amount and applicable to the amboceptors of a considerable number of other species. Complement does not appreciably change in amount by the ordinary processes of immunization.

Immune Hetero-hemolysins.—Hemolytic amboceptors may be natural to a blood or may be developed by immunization. Autolysins and isolysins may be produced but with great difficulty and variability. Isolysins may be present normally, notably in man. Heterolysins may be found normally and can be readily produced by artificial immunization. Bordet produced heterolysins by intraperitoneal injection of erythrocytes. They may also be induced by the subcutaneous and by the intravenous routes of inoculation. Two important conceptions of the mode of action of the amboceptor have been proposed. Bordet, Metchnikoff and the French school consider the action to be in the nature of a sensitization or fixation of the antigenic cells, so that they are more readily acted on by the complement, in somewhat the same fashion that a mordant prepares a cell so that it will stain more readily. Ehrlich and Morgenroth and the German school consider the amboceptor as a link which brings together antigen and complement; in other words, in their conception the amboceptor possesses two binding groups, a cytophilic and a complementophilic group, each capable of acting as a specific receptor. In order to discuss the various theoretical considerations more clearly, it is essential that the well-established facts in regard to hemolysis be presented as they are ordinarily demonstrated in a practical way.

Preparation of Immune Hemolysins. The Blood Antigen.—In immunization for the production of a hemolysin it is necessary to select the animals to be used. The rabbit is usually chosen as the animal to be immunized because of the fact that it is easily available, relatively inexpensive, and yields a fairly large amount of blood. In selecting the animal whose blood-corpuscles are to be used for the production of hemolysin, convenience again plays a part. The sheep is the animal most commonly employed, although the goat is equally useful. Reasonably large amounts of blood can be secured from such animals at short intervals of time, without deleterious effects. Dog blood is unsatisfactory because the corpuscles do not resist standing for any length of time. The cat is undesirable because of its relatively small size. In order to secure blood from a goat or sheep the animal is either strapped on a board, or may be held by a skilled attendant. The neck is shaved over the jugular vein, the area washed with soap and water, cleansed with alcohol, and the vein distended by pressure over the jugular bulb at the base of the neck. The blood is collected through a fairly large needle into a sterile flask containing glass beads or fragments of glass tubing. Rotation of the flask or shaking during the collection and continued shaking for five or ten minutes after the collection completely defibrinates

the blood. The blood may be injected as defibrinated blood for purposes of immunization, but as a rule in order to avoid any influences the serum may have, the blood is washed so that only corpuscles are injected. For purposes of washing 50 c.c. centrifuge tubes are desirable, but if these are not obtainable, the 15 c.c. size may be employed. The blood is measured into the tube with a pipette, usually to the amount of 5.0 c.c. The amount of blood is marked with a grease pencil and the tube filled with physiologic salt solution. The tube is centrifuged until the blood is thrown down. The supernatant fluid is poured off and the tubes again filled with salt solution. The sedimented corpuscles are shaken up into the salt solution and again centrifuged. This operation is repeated again, and the blood is said to have been washed three times. After the last centrifugation the supernatant fluid is poured off and the sedimented blood-corpuscles restored to original volume by addition of salt solution. In order to make a five per cent. suspension, this is washed into a 100 c.c. cylinder and made up to 100 c.c. volume with salt solution. Any other percentage desirable may be made by appropriate additions of salt solution to the original blood mass.

Preparation and Collection of Immune Sera.—The injection into the rabbit may be by subcutaneous, intraperitoneal, or intravenous routes. The intravenous route produces immune bodies most rapidly, as has been shown by Bullock, and as a rule produces an immune serum of higher titer than is obtainable by other methods. An excellent way to produce hemolysin rapidly is to inject intravenously into the rabbit three doses 4.0 c.c. each of 50 per cent. suspension of washed sheep or goat erythrocytes at intervals of five days. The method of intravenous injection has previously been described in connection with the production of agglutinins (see page 82). A test bleeding may be made from the posterior ear vein five to seven days after the last injection, and if the titer of the serum is not sufficiently high, one or two more injections may be given. When sufficiently high the rabbit is bled from the femoral artery as previously described (see page 83). The blood is collected in a flask, the flask inclined at an angle of about 45° until the blood is firmly clotted. The flask is then placed in an upright position in the refrigerator for about twenty-four hours, after which the collected serum is pipetted into a sterile container. Melick, in a study of the influence of colloidal suspensions on the production of hemolysis, finds that if he gives preliminary intravenous injection of aleuronat suspension and subsequently immunizes with blood-corpuscles, the hemolytic sera are of considerably higher titer than in animals not so treated.

Titration of Immune Sera.—For titration of the hemolysin in the rabbit serum, it is necessary to have a 5 per cent. suspension of the antigenic corpuscles, the serum to be tested and in addition fresh guinea-pig serum which serves as complement. In such a titration the 5 per cent. suspension of corpuscles and the complement are regarded as standards and employed in the same doses throughout the series of tubes. If 0.5 c.c. of serum and 0.5 c.c. of corpuscle suspension are employed, 0.05 c.c. of complement is usually sufficient. Before attempting the titration the rabbit immune serum should be inactivated by heating in a water bath at 56° C. for one-half hour. Dilutions of the inactivated immune amboceptor are made as a rule 1-10, 1-100, 1-500, 1-1000, 1-1500, 1-2000, 1-2500, 1-3000, 1-4000. The guinea-pig serum (complement) is diluted 1-10. The following protocol will show how the series of tubes is set up:

5% suspension sheep cells	Amboceptor		Complement 1-10	Result
0.5 c.c.	1-100	0.5 c.c.	0.5 c.c.	CH
0.5 c.c.	1-500	0.5 c.c.	0.5 c.c.	CH
0.5 c.c.	1-1000	0.5 c.c.	0.5 c.c.	CH
0.5 c.c.	1-1500	0.5 c.c.	0.5 c.c.	CH
0.5 c.c.	1-2000	0.5 c.c.	0.5 c.c.	CH
0.5 c.c.	1-2500	0.5 c.c.	0.5 c.c.	CH
0.5 c.c.	1-3000	0.5 c.c.	0.5 c.c.	PH
0.5 c.c.	1-4000	0.5 c.c.	0.5 c.c.	N
0.5 c.c.	0.5 c.c.	0.5 c.c.	N
0.5 c.c.	1-100	0.5 c.c.	N

The last two tubes are controls and should be made up to volume by addition of 0.5 c.c. saline in place of amboceptor in the one and of complement in the other. The letters CH indicate complete hemolysis, PH partial hemolysis, and

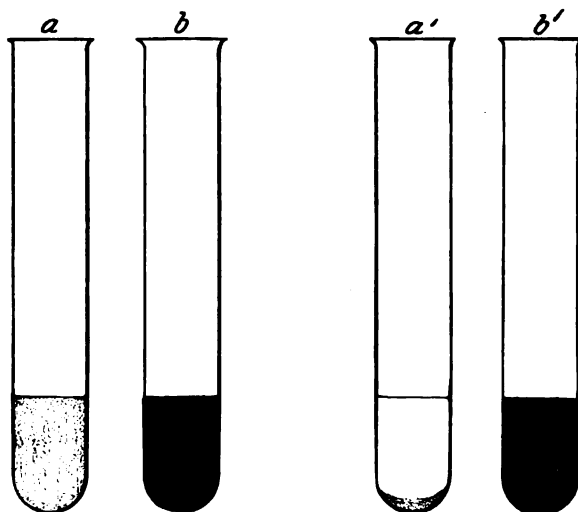


FIG. 12.—*a* shows a saline suspension of blood-corpuscles before hemolysis; *b* the same after hemolysis. *a'* and *b'* present the appearance of *a* and *b* after sedimentation of corpuscles. From Noguchi, Serum Diagnosis of Syphilis.

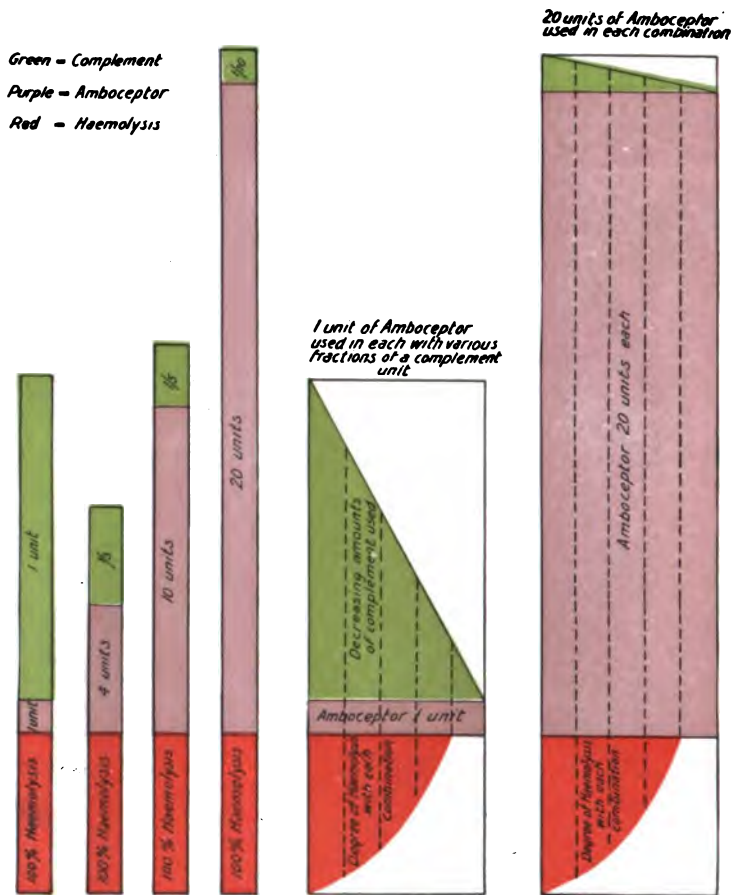


FIG. 13.—Diagrams showing relative proportions of reagents in hemolysis. The group of four columns shows the reduction possible in amounts of complement with increasing amounts of hemolytic amboceptor, in the production of complete hemolysis. The second and third parts of the figure show the curve of reduction in percentage, or degree, of hemolysis following reduction in amount of complement in the presence of constant quantities of amboceptor. From Noguchi, Serum

Diagnosis of Syphilis.

N no hemolysis, the reading being made after a period of incubation in the water bath at 37° C. This period may be thirty minutes, one hour or two hours, but subsequent experiments with the same system of amboceptor, complement and corpuscles must be made with the same period of incubation as practised in the original titration. In this laboratory one hour is the standard time for incubation. In order to make results somewhat more clear-cut, the rack of test tubes may be placed in the refrigerator over night and the results read the following morning. The lapse of twelve or eighteen hours time permits the corpuscles to settle to the bottom of the test-tube; therefore any red coloring of the supernatant fluid may be interpreted as a partial or complete hemolysis, depending on the depth of color and the amount of sediment remaining on the bottom of the tube. The controls which are used in this experiment demonstrate that neither complement nor inactivated amboceptor will produce hemolysis. The result given in the above experiment indicates that at some point between the dilutions 1-2500 and 1-3000 the exact end point of titration is to be found. In order to determine the exact end point it is well to set up an additional series with dilutions of 1-2500, 1-2600, 1-2700, 1-2800, 1-2900, and 1-3000 with the necessary controls. If it is found that complete hemolysis takes place in a dilution 1-2700 and not in the dilution 1-2800 the dilution 1,2700 is taken as the end point or titer. The unit of amboceptor therefore is 1-2700 of 0.5 c.c. or 1-5400 of 1 c.c. In the experiment outlined above, the unit of amboceptor would be designated as 0.5 c.c. of a 1-2700 dilution of the immune serum.

Titration of Complement.—As has been indicated previously, the amount of complement in guinea-pig serum varies in different animals. Therefore subsequent experiments with this amboceptor must be controlled by titrating the complement. This may be done by setting up a series of tubes as follows, the control tubes being made up to volume with salt solution:

Erythrocytes suspension	Amboceptor 1-2700	Complement 1-10	Result
0.5 c.c.	0.5 c.c.	0.5 c.c.	CH
0.5 c.c.	0.5 c.c.	0.4 c.c.	CH
0.5 c.c.	0.5 c.c.	0.3 c.c.	PH
0.5 c.c.	0.5 c.c.	0.2 c.c.	N
0.5 c.c.	0.5 c.c.	0.1 c.c.	N
0.5 c.c.	...	0.5 c.c.	N
0.5 c.c.	0.5 c.c.	...	N
0.5 c.c.	N

In this experiment it is found that 0.4 c.c. of the new complement is sufficient for activating the unit of amboceptor. Therefore, whereas in the first experiment 0.5 c.c. 1-10 complement dilution was the unit of complement, in the second experiment 0.4 c.c. 1-10 dilution complement is the unit. If it is found that in none of these tubes complete hemolysis takes place because of weak complement, it will then be necessary to set up an additional series with complement diluted 1-5 instead of 1-10.

Quantitative Relations of Amboceptor and Complement.—The quantitative relationship between the amount of complement and amboceptor used has been very extensively studied. It is now known that a larger amount of complement will require a smaller amount of amboceptor for the production of complete hemolysis in the standard blood-corpuscle suspension and conversely a smaller amount of complement requires a larger amount of amboceptor. Thus, if we use two units of complement, hemolysis will occur in the presence of less than one unit of amboceptor. If we use two units of amboceptor, it will require less than one unit complement to produce complete hemolysis. This relationship, however, is not in definite proportion. For example, if four units of amboceptor are employed, one-third unit of complement is necessary. This relationship is beautifully illustrated in the diagram (Fig. 13) taken from Noguchi.

Quantitative Relations of Amboceptor and Antigen.—By subsequent studies of different mixtures, it was found that the unit of standard corpuscle suspension can take up considerably more than one unit of amboceptor. This amount varies with the total quantity of immune body present. For example, Muir found that on addition of twelve doses of amboceptor one dose remained free, on addition of sixteen doses of amboceptor two doses remained free, on addition of twenty doses three doses remained free and on the addition of twenty-three doses of amboceptor, four doses remained free. When, however, the mixture of complement, amboceptor and red blood-corpuscles is properly adjusted, the reaction completely uses up the amboceptor, complement and, by hemolysis, all the red blood-corpuscles. Correspondingly, if two units of complement are employed in the presence of one unit of amboceptor, it does not follow that after the reaction one unit of complement will remain free. As a matter of fact, practically the entire two units of complement will be utilized in the reaction. Nevertheless, increasing the amounts of complement will leave more and more complement free in the supernatant fluid. These points will be made somewhat clearer after subsequent experiments have been outlined.

Relative Affinities of Amboceptor and Complement.—In the introductory paragraph it was pointed out that the amboceptor has a special affinity for the antigenic red blood-corpuscles, but that the complement has no such affinity. This is illustrated by the fact that when red blood-corpuscles are set up against amboceptor they will absorb the amboceptor, but if they are set up in the presence of complement they will not absorb complement.

The following experiment illustrates this point: Two centrifuge tubes are marked A and B. In tube A are placed 1.0 c.c. standard erythrocyte suspension (5 per cent. suspension) and 1.0 c.c. inactivated immune serum so diluted as to contain two units amboceptor. This tube is incubated at 37° C. for thirty minutes and then centrifuged. The supernatant fluid is pipetted into a tube marked A 2. The erythrocyte sediment in tube A is washed in salt solution, again centrifuged and the supernatant fluid discarded. The sediment in tube A is resuspended in 1.0 c.c. salt solution and two units of complement, i.e., 1.0 c.c. 1-10 dilution are added. To tube A 2 are added two units complement (1.0 c.c. 1-10 dilution) and 1.0 c.c. 5 per cent. erythrocyte suspension. These tubes are incubated for one hour at 37° C. Tube A will show hemolysis because the sedimented corpuscles have absorbed amboceptor and the addition of complement is sufficient to complete the reaction. Tube A 2 will not show hemolysis because the amboceptor is not in the supernatant fluid and the complement is not sufficient to lysis the added corpuscles. At the same time the converse of the foregoing experiment may be conducted. In tube B are placed 1.0 c.c. standard erythrocyte suspension (5 per cent. suspension) and 1.0 c.c. fresh guinea-pig serum diluted 1-10. This is incubated thirty minutes at 37° C., centrifuged and the sediment washed. The supernatant fluid is placed in tube B 2. The sediment in tube B is resuspended in 1.0 c.c. salt solution and 1.0 c.c. immune serum so diluted as to contain two units amboceptor is added. To the supernatant fluid in tube B 2 are added 1.0 c.c. immune serum (two units amboceptor) and 1.0 c.c. erythrocyte suspension. These tubes are incubated for one hour at 37° C. Tube B 2 will show hemolysis because the supernatant fluid after the centrifugation still contains complement, so that the addition of amboceptor and erythrocytes permits of completion of the reaction. Tube B will not show

hemolysis because the corpuscles in the sediment have not taken up any complement, and the addition of amboceptor is not sufficient for the reaction to occur.

Selective Absorption of Amboceptor and Complement.—Not only is it possible to show, as has been done in the preceding experiment, that red blood-corpuscles will combine with amboceptor and not with complement, but if conditions are so arranged that hemolysis is prevented, it is possible to demonstrate that red blood-corpuscles will selectively absorb amboceptor from a mixture of amboceptor and complement. In order to prevent hemolysis, it is necessary to permit the absorption to take place at 0° C. Not only must this precaution be observed, but the tubes must be cooled, the various reagents in the mixture must be cooled in advance and the centrifuge carrier must also be cooled.

The various reagents are placed in test tubes and all the tubes placed in a mixture of salt and ice. Into a cold centrifuge tube are placed 1.0 c.c. 5 per cent. erythrocyte suspension, 1.0 c.c. inactive immune serum so diluted as to contain two units amboceptor and 1.0 c.c. guinea-pig serum, 1-10 dilution. This tube remains in the salt-ice mixture for thirty minutes and is then centrifuged. The supernatant fluid is poured off and divided so that one-half the amount is placed in each of two tubes. The sediment is washed in cold salt solution and resuspended in 4.0 c.c. cold salt solution. These 4.0 c.c. are divided between two tubes. The four tubes so prepared are set up as follows:

TUBE 1	
Supernatant fluid	1.5 c.c.
Fresh guinea-pig serum, 1-10	0.5 c.c.
5 per cent. erythrocyte suspension	0.5 c.c.

TUBE 2.	
Supernatant fluid	1.5 c.c.
Immune rabbit serum	0.5 c.c.
5 per cent. erythrocyte suspension	0.5 c.c.

TUBE 3.	
Sediment	2.0 c.c.
Fresh guinea-pig serum, 1-10	0.5 c.c.

TUBE 4.	
Sediment	2.0 c.c.
Immune rabbit serum	0.5 c.c.

These tubes are incubated for one hour at 37° C. Inasmuch as the supernatant fluid no longer contains amboceptor, tube 1 will fail to show hemolysis, but in the case of tube 2 the amboceptor is added, and since the supernatant fluid contains complement which has not been absorbed by the corpuscles, hemolysis will result. The sediment has absorbed amboceptor from the mixture; therefore, in the case of tube 3, the addition of fresh guinea-pig serum will serve to produce hemolysis. The sediment has not, however, taken up any complement, the addition of the immune serum in tube 4 will not serve to complete the reaction, and hemolysis will not occur. By the use of sera containing other hemolytic amboceptors, it is possible to show not only that absorption of amboceptor may occur from a complement amboceptor mixture, but that this absorption is specific for the particular amboceptor concerned.

Influence of Amount of Complement.—Although, as will be shown subsequently, the concentration of complement plays a part in the com-

pletion of hemolysis it can be demonstrated that the absolute amount rather than the degree of concentration is of importance in regard to the amboceptor.

This may be shown by placing in each of four tubes 0.5 c.c. 5 per cent. suspension of corpuscles and adding to the second, third and fourth tubes, respectively, four, nine and fourteen volumes of salt solution. To each of the four tubes is added one unit amboceptor, and the mixture incubated at 37° C. for one-half hour to permit absorption of amboceptor. The tubes are centrifuged and the supernatant fluid is discarded. To each tube is added 1.0 c.c. complement so diluted as to contain one unit, and the mixtures again incubated at 37° C. for one hour. Hemolysis will occur equally in all tubes showing that the complete absorption of amboceptor by the cells occurred in spite of marked dilution in some of the tubes.

Rate of Absorption of Amboceptor.—The absorption of amboceptor varies in rapidity under different conditions. For example, absorption takes place more readily at 37° C. than at 20° C., and more readily at 20° C. than at 0°. An exception to this rule appears in cases of paroxysmal hemoglobinuria. Some of these cases possess in the blood an autohemolysin which does not enter into combination with erythrocytes at body temperature. If the blood is withdrawn and placed at a temperature of 0° to 10° C. for an hour the cells absorb the amboceptor and subsequent incubation at 37° C. permits the interaction of complement so that hemolysis results. With this and possibly some other exceptions the general rule holds true that temperatures approaching 37° C. favor the union of amboceptor and antigen. Certain physical conditions also play a part in rapidity of absorption as may be shown by the following experiment in which the mixture of corpuscles and amboceptor is made under different conditions.

Two wide test tubes or small beakers are marked A and B. In A are placed six units of cell suspension; namely, 3.0 c.c. 5 per cent. suspension. To this are added drop by drop 3.0 c.c. amboceptor, so diluted that it contains six units, the tube being shaken constantly during the addition. In tube B the process is reversed, the amboceptor being placed in the tube and the cell suspension added drop by drop. These mixtures may be titrated against varying amounts of complement in a series of tubes, or six units of complement may be added to tube A and tube B. An hour's incubation at 37° C. will show less active hemolysis in tube B than in A. The probable explanation is that the first cells added to the amboceptor in tube B absorb all or nearly all the amboceptor, and the subsequently added cells are only partly saturated or take up no amboceptor at all.

This experiment illustrates the very rapid absorption of amboceptor by cells and also the fact that cells may absorb considerably more than one unit of amboceptor.

Dissociation of Amboceptor-Antigen Union.—Whereas temperatures up to 37° C. appear to favor absorption of amboceptors, Bail, Tsuda and others have shown that a temperature of 42° C. results in a partial dissociation of amboceptor. That dissociation of amboceptor and cells could occur was shown independently by Muir and by Morgenroth. Muir mixed 1.0 c.c. 5 per cent. corpuscle suspension with ten units amboceptor and allowed the mixture to stand at room temperature for one hour. The tube was then centrifuged, the corpuscles

washed three times and resuspended in salt solution to a volume of 1.0 c.c. To this was added 1.0 c.c. untreated corpuscle suspension, the tube shaken and placed at 37° C. for one hour. At the end of this time four units of complement were added, the tube incubated again for one hour and complete hemolysis was found. Thus it was found that the original cells yielded at least one unit of amboceptor for the new cells. Although in the report cited on page 120, twelve units gave one free unit, Muir states that usually one unit of amboceptor can be obtained from corpuscles containing six units. In this experiment the dissociation was at 37° C., but dissociation takes place at room temperature, although more slowly, and at 0° C. it is practically nil. By working with sensitized cells and with supernatant fluids, it is possible to titrate the latter so as to determine the exact quantities of amboceptor dissociated. Kosakai, in working with so-called pure hemolysins, has recently shown that the antigen and amboceptor union is reversible to a greater extent than has previously been supposed. He maintains that the reversibility under these circumstances is almost or quite complete.

Specificity of Amboceptors. Group Reactions.—The hemolytic amboceptors are highly specific, but show, as do other immune bodies, group reactions. Ehrlich and Morgenroth showed that immune sera prepared against ox blood are hemolytic also for goat and sheep blood and that a hemolysin prepared against goat blood also dissolves ox blood. Marshall showed that an antihuman hemolysin acts on monkey blood and vice versa. In any case the hemolysin is most active in the presence of the antigenic corpuscles. Treatment of a hemolytic immune serum with heterologous corpuscles removes more of the specific immune body than is the case in other group reactions. For example, Muir developed an anti-ox-blood serum which, in a dose of 0.0005 c.c. dissolved 1.0 c.c. 5 per cent. suspension ox corpuscles and, in a dose of 0.0012 c.c. dissolved a similar suspension of sheep corpuscles. Absorption by sheep corpuscles in excess reduced the titer against ox corpuscles so that the serum dissolved the latter in doses of 0.0012 c.c.; in other words, the titer of the serum was reduced to about half its original strength. Ehrlich and Morgenroth showed that if the quantity of sheep corpuscles is carefully adjusted so as exactly to equal the hemolytic power for such corpuscles, the fraction of amboceptor lytic for sheep corpuscles may be absorbed without reducing the titer against ox cells. If, however, the amboceptor for ox blood is removed by absorption with ox corpuscles the hemolytic power for sheep corpuscles is entirely destroyed. Thus it is seen that there is close similarity with the group reactions of agglutinins and other immune bodies. Ehrlich and Morgenroth explain the phenomenon by assuming that each amboceptor contains numerous "partial amboceptors" formed in the immune animals in response to relatively undifferentiated receptors of the antigenic cells. In other words, ox-blood corpuscles are supposed to contain a certain number of receptors specific to those cells, and in addition other receptors that are closely similar to or identical with

certain receptors of sheep cells and goat cells. Therefore, the injection of ox cells leads to the production of an amboceptor containing partial amboceptors specific for ox blood and partial amboceptors specific for the common receptors of ox, sheep and goat cells. The removal of the partial amboceptors common to all three cell receptors will not remove that specific for ox cells, but ox cells will remove both the specific and common fractions. This explanation has been the subject of much experiment, particularly with anti-hemolysins, and modern views are not entirely in accord with the original views of Ehrlich. The subject will be referred to again in connection with a discussion of anti-amboceptors and anti-complements. In the same place will be found a discussion of the interpretation of the amboceptor as made up of a cytophilic and complementophilic group.

Nature of the Antigen.—In ordinary practice the entire erythrocyte is employed for immunization, but attempts have been made to determine what fraction of the cell is truly antigenic. Ford and Halsey have shown that the use of either stroma or the laked hemoglobin may serve to produce hemolysins, but they obtained only questionable results following the use of pure hemoglobin. Stewart obtained essentially the same results. Nucleo-proteins obtained from dog blood are capable of producing specific hemolysins. Pearce and his co-workers have shown that nucleo-proteins from washed organs also lead to the formation of hemolysins specific for the homologous species. Organ and cell extracts free from blood also serve as hemolysinogens; the best example is an extract of spermatozoa, for in this instance there is no question of blood contamination of the extract. Of further interest is the fact that ether extracts of erythrocytes, alcohol-ether extracts, and extracts in 1.5 per cent. sodium bicarbonate induce the formation of weak hemolysins without the coincident formation of hemagglutinins. This indicates that the hemagglutinin and hemolytic amboceptor are probably separate and distinct antibodies.

Nature of the Amboceptor.—The amboceptor, although it resists heat of 56° for one hour or more, is injured by heat of 60° C. for twenty minutes, is almost completely destroyed by 70° C. for one hour and is completely destroyed by boiling. Like antitoxin, it does not dialyze, is electro-positive and is resistant to ultra-violet rays. It is carried down in the euglobulin fraction of the serum protein, but by various methods of purification may be obtained in an almost protein-free state. The method of purification described by Kosakai is of importance from various points of view and deserves some description at this point. He requires a hemolytic serum which titrates 1–10,000. This is diluted to 100 times its volume with salt solution and 5 c.c. of the diluted serum are poured into 4 c.c. blood-cell suspension. The union of amboceptor and red cells is accomplished by exposure at room temperature for fifteen to twenty minutes, after which the cells are freed from serum by repeated washing. To the antigen-amboceptor combination is added isotonic or slightly hypertonic aqueous solution of a sugar such as saccharose, glucose or lactose, and the mixture incu-

bated at 55° C. for fifteen to twenty minutes, during which period it is shaken several times. The mixture is centrifuged and the supernatant fluid placed in a separatory funnel with five to ten volumes of ether and shaken for one or two hours until the solution becomes quite colorless. The saccharose solution is separated from the ether and dialyzed in running water in order to free it from sugar and salt. After dialyzation the solution is concentrated in a vacuum until it reaches the original volume of blood serum employed. Strong salt solution may prevent amboceptor from entering into combination with complement, but it does not interfere with the amboceptor cell union. Alkalis may prevent either form of union and may serve partly to dissociate amboceptor cell combinations.

Mechanism of Operation of the Amboceptor.—As has been pointed out previously, the action of amboceptor is differently interpreted by the Ehrlich and the Bordet schools. If the Ehrlich view of the two-fold binding group is to be adhered to, it should be possible to show on the one hand a combination with antigen, and on the other a combination with complement. Of these possibilities there is no doubt that combination with cells is possible, but as yet no conclusive evidence has been produced to show a combination between complement and an amboceptor not united to its antigen. The discovery of the Neisser-Wechsberg phenomenon (see page 147) was regarded as demonstrating a combination between free amboceptor and complement. This explanation, however, does not take into account the possible relationship to certain colloidal reactions such as have been described in connection with the inhibition zone of strong agglutinins and is therefore not to be regarded as settled. Ehrlich and Morgenroth stated that if amboceptor is repeatedly injected into animals an anti-amboceptor is produced which serves to combine with the cytophilic group of amboceptor, but Bordet found that a normal serum, free from hemolytic amboceptor could be used to produce the same immune body, and argued therefrom that this antibody could not be regarded as a specific receptor. Ehrlich and Sachs admitted the fact of Bordet's experiments and came to the conclusion that the substance is anti-complementophile, rather than anti-cytophile. As will readily be seen this argument presupposes the correctness of the Ehrlich conception of amboceptor, and is therefore not to be accepted as conclusive. Without the actual demonstration of the union of free amboceptor and complement, the union of antigenic cells and amboceptor is of quite as much value in support of the Bordet view of sensitization as in support of the Ehrlich hypothesis. Nevertheless, Ehrlich and Sachs have reported what they believe to be a crucial experiment in that it appears to show that at least in some instances free amboceptor and complement may combine. Horse serum is slightly hemolytic for guinea-pig erythrocytes and ox serum is somewhat more so. If inactivated ox serum and fresh horse serum are added to guinea-pig cells, hemolysis occurs, the ox serum acting presumably as an amboceptor, the horse serum as complement. If the guinea-pig cells are treated with inac-

tivated ox serum for a time ordinarily sufficient for amboceptor absorption, washed free of serum and then treated with fresh horse serum as a complement, no hemolysis occurs. Furthermore, under these conditions no hemolytic immune body has been absorbed from the ox serum. Hemolysis only occurs when fresh horse serum and inactivated ox serum are added as a mixture. The interpretation is that in this particular hemolytic system the amboceptor must be combined with complement before the amboceptor combines with the cells, or, in other words, that the complementophilic group of a free amboceptor has united with complement independently of the cytophilic group.

Conglutinin.—Bordet and Gay have studied the phenomenon described in the preceding paragraph and have come to a different conclusion as to interpretation because of their discovery of a so-called "bovine colloid" in the ox serum. They attribute the hemolysis in the Ehrlich-Sachs phenomenon almost entirely to the amboceptor and complement of the horse serum. The complex of guinea-pig cells and the two bodies in the horse serum serves to attract the bovine colloid which augments the complementary action of the horse serum so as to produce complete hemolysis and at the same time produces marked agglutination of the cells. This colloid is thermostable, is probably of protein nature, unites with a complex of cells, amboceptor and complement, but does not act upon either normal cells or cells saturated with amboceptor. Bordet and Streng in a later study named the colloid "conglutinin." Streng found that the same phenomenon could be demonstrated in regard to bacteriolysis and that conglutinin is present in the sera of the ox, goat, sheep and certain other herbivora but not in the sera of the cat, dog, guinea-pig, or bird. Sachs and Bauer have not offered a better explanation of the phenomenon unless the German theory of amboceptor is unqualifiedly accepted. In our opinion both sides of this controversy deserve the most careful consideration and much light may be thrown by further study. The more modern views of immunological processes, influenced as they are by the great advances in colloidal chemistry, tend toward acceptance of the Bordet hypothesis of sensitization of antigen by the thermostable constituent of cytolytic sera, at least until and unless more conclusive contradictory evidence can be produced.

Complement. Distribution.—Complement is that thermolabile element of normal blood which in the presence of amboceptor and antigen completes the cytolytic reaction. As regards hemolysis, complement in the presence of hemolytic amboceptor causes solution of the red blood-corpuscles and thus renders the reaction visible. Complement is found in the blood and in lesser amount in nearly all the other body fluids except the aqueous humor of the eye. It is also found in inflammatory exudates and sometimes in transudates, but it is not present in the urine, nasal secretion or the secretion of other glands except that of the breast (milk). The amount in the blood is fairly constant for any given individual, but during the first twenty-four hours after

birth the complement content of the blood has been found to be rather small; Gay has found it to be somewhat less in women than in men. Moro has found it to be less in bottle-fed than breast-fed babies. Although individual variation may be great, there is a certain uniformity in different members of the same species. This is true throughout a large number of species, except the horse, in which species it is found to vary markedly. Different species as such contain different amounts of complement. The guinea-pig contains, as a rule, more complement per cubic centimeter than other species. Man and rabbit contain less than the guinea-pig, and in the case of the mouse it is very difficult to demonstrate any complement at all. It has recently been found that insects and mollusks contain practically no complement.

Alterations of Amount of Complement.—The amount of complement in a given blood may be made to vary by artificial means. For example, the injection of indifferent materials, such as foreign blood plasma, bouillon, aleuronat, pepton, yeast, nuclein, physiological salt solution, produces an increase in the amount of complement, but this increase is not permanent. Similarly complement may be increased for a short time following the injection of pilocarpin, phlorizin, staphylococci, oil of turpentine and thyreoidin; exposing an animal to high temperatures may also increase complement. Although it is generally true that complement is not increased by immunization, nevertheless Cantacuzène has recently shown that by injecting red blood-corpuscles into certain marine invertebrates he is able to increase the amount of complement in their blood. Complement may be reduced temporarily by the injection of sodium taurocholate, potassium picrate, toluylendi-amin and more permanently by experimental phosphorus poisoning, experimental chronic suppuration, starvation and by alcohol poisoning. If sensitized blood-cells, *i.e.*, blood-cells saturated with amboceptor, are injected into an animal, it can be demonstrated that the amount of complement is reduced by the hemolysis which takes place *in vivo*. Shaw has found that in the case of recently acquired syphilis, although the blood before treatment shows no alteration of complementary activity, yet the administration of salvarsan may reduce this activity to a considerable degree. The experimental investigations of the effect of disease in man on the complement content of his blood are very unsatisfactory because human blood normally contains only a small amount of complement and the detection of any variation is susceptible to a wide margin of experimental error.

Method of Obtaining Complement.—Complement is usually obtained from the guinea-pig, although under special circumstances it may be obtained from other animals. The blood may be withdrawn in any manner adapted to such a procedure. In the case of the guinea-pig the method employed in this laboratory is to anesthetize the animal very slightly, pull the hair from the neck, make a longitudinal slit in the mid-line of the neck, place a 15 c.c. centrifuge tube toward the upper end of the slit with its lip firmly pressed into the opening, then with a scissors snip the carotid artery, carefully avoiding the trachea. The animal is then held head downward while the blood drains into the tube. The blood is allowed to clot in the tube and the clot separated from the side of the tube with a long sterile or clean needle, as the necessity of the case indicates. The clot separates best at room temperature, but if centrifuga-

tion cannot be done immediately the clot may be allowed to separate in the ice chest. As soon as the serum has separated out of the clot, the tube is centrifuged and the serum collected by means of a pipette. If guinea-pigs are large, the blood may be collected in smaller quantities by heart puncture or by bleeding from an ear vein, thus obviating the necessity for killing the animal.

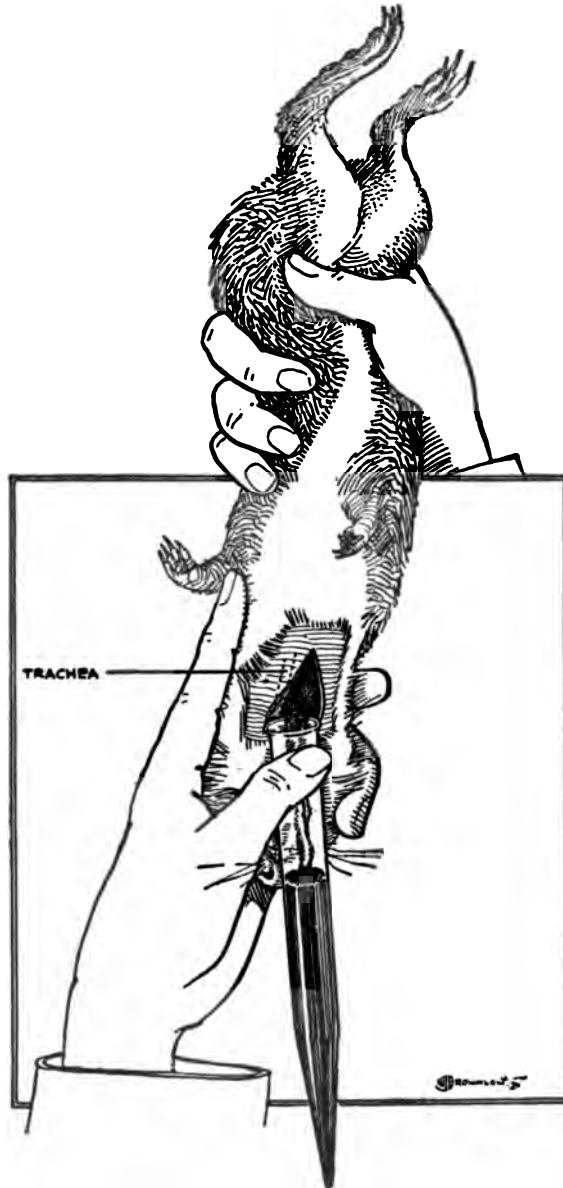


FIG. 14.—Method of obtaining blood from guinea-pig (See text).

Origin of Complement.—Considerable controversy has been waged concerning the origin of complement since the time that Hankin and

subsequently Metchnikoff expressed the belief that complement originates in the leucocytes of the body and is only liberated upon the death of these cells. Metchnikoff used the term *cytase* to indicate what we now call complement and believed that the microphages gave rise to a microcytase capable of dissolving blood and other body cells. Pfeiffer and certain other German workers take a diametrically opposed position and maintain that the leucocytes furnish none of the complement in the blood. A. von Wassermann and also Landsteiner believe that the leucocytes may constitute one source of origin for the complement, and it seems practically certain from modern investigations that several organs play a part in the formation of complement. Before the bactericidal action of blood was thoroughly understood as due to the interaction of amboceptor and complement, certain studies seemed to indicate that exudates rich in leucocytes were active as bactericidal agents, but it is now understood that other constituents of the exudate take part in this phenomenon and more recent experiments show that extracts of leucocytes do not yield a complement. It has been shown further that variations in the total leucocyte count in an animal produce no corresponding variations of complement content. Neufeld and certain others take the view that even inside the living leucocytes there is no complement because they have found that destruction of red blood-corpuscles within living leucocytes takes place at a distinctly slower rate of speed than is the case in ordinary hemolysis. Furthermore, they point out that the method of destruction is quite different, in that ordinary hemolysis shows simply liberation of hemoglobin without destruction of the stroma. Metchnikoff's belief that the death of the leucocytes yields complement was supported by an experiment which apparently showed that complement is present in serum after clotting, but not in plasma. A considerable amount of experimental evidence has been adduced, since this statement of Metchnikoff, to show that plasma contains complement in the same amount as does serum. Some of these experiments appeared to be invalid on the ground that immunological work with a plasma is likely to lead to coagulation, thus producing a serum for the actual experiments. After these objections had been presented, further experiments were performed which overcame such objection, and it now seems perfectly clear that plasma contains complement. This fact has been firmly established by the recent work of Watanabe.

Nature of Complement.—Complement is probably of protein nature, inasmuch as it is destroyed in coagulation of the serum by heat and is digested by trypsin. Noguchi and his co-workers were of the opinion that complement is a combination of soap and a protein, but numerous other workers failed to confirm these studies. This statement of Noguchi, as well as the work of Kyes, with cobra venom led to the hope that it might be possible to prepare an artificial complement. Landsteiner and Jagic have investigated the question and have shown that whereas it is possible to substitute for amboceptor a colloidal solution of silicic acid, which nevertheless shows none of the specific

characters of amboceptor, it is absolutely impossible up to the present time to offer any substitute for complement. Complement resembles an enzyme in that it is thermolabile, disintegrates cells, does not pass through Berkefeld filters, is adsorbed by kaolin and destroyed by shaking. Furthermore, it activates amboceptor much in the same manner as entero-kinase activates trypsinogen. As against the idea that complement is an enzyme is the fact that in the reaction of hemolysis, hemoglobin is liberated without destruction of the stroma of the cells and the further fact that complement acts quantitatively, following in a general way the law of multiple proportions. As is well known, heat at 56° to 60° C. for one-half hour destroys the complementary activity of a serum. It has recently been shown, however, that if heat of 56° C. is applied for only a short period, *i.e.*, from seven to ten minutes, the complementary action is restored after several hours have elapsed (the phenomenon of Gramenitski). This is interpreted as due to an agglomeration or aggregation of protein particles resembling heat coagulation of protein. The restoration of activity after standing is ascribed to a dispersion of the protein aggregates so that they can act nearly or quite as they did originally. Ultra-violet rays destroy complement, but it is stated that X-rays do not. Recent work in this laboratory by Ecker has shown that the visible spectrum also serves to reduce complementary activity. Experimental conditions in this instance made it possible to work with three divisions of the spectrum, namely, a division near the violet end, a division in the middle of the spectrum and a division near the red end. It was found that those rays toward the violet end of the spectrum were more active than the rays in the middle of the spectrum and the latter were more active than the rays at the red end of the spectrum. That this is a function of the wave-length of the ray is not absolutely certain but seems probable in view of the work of Bovie, Brooks and others, which shows that the presence of cells in the serum reduces the activity of the ultra-violet rays. That the destruction, however, is a function of the penetrability of the rays is not borne out by the statement that X-rays fail to destroy complement. We have also been able to show in this laboratory that drying of complement produces some deterioration. Other workers have stated, however, that if the complement is mixed with a proper concentration of salt, preferably about 8 per cent. and then dried, the salting nullifies the destructive action of desiccation and the dried serum under these circumstances may be preserved for a considerable period of time. Complement may be inhibited by the presence of hydroxyl ions but is restored to activity by the addition of hydrogen ions. Complement can be made to combine with magnesium, calcium, barium, strontium and sulphate ions and can be separated by simple chemical precipitation. Acids and alkalis in sufficient concentration also serve to destroy complement.

Preservation of Complement.—Owing to the extreme lability of complement, the question of prolonged preservation assumes considerable importance. The fresh serum may be desiccated in air, in

vacuum, in vacuum after freezing, or on filter paper. In the hands of certain workers various methods of this sort have proven more or less successful but do not seem to be widely applicable. It is of importance to keep in mind that under such conditions the desiccation of serum does not remove the possibility of the destructive action of light. Other methods of preservation include salting with sodium chloride and also with sodium acetate. The former has been fairly successful, but the latter has been completely abandoned. Another method is salting and then freezing, but this has been found to be in no way superior to freezing without salting. According to Bigger, it is of extreme importance that the serum should be sterile to ensure the success of any method of preservation. Browning and Mackie have found that frozen serum kept at a temperature of -15° C. retains its complementary power three months without appreciable loss. Noguchi and Bronfenbrenner found that at 10° C. the serum loses one-half its original strength at the end of twenty-four hours. If it is kept at 37° C. it loses two-fifths of its strength at the end of six hours; at 45° C. one-half hour exposure reduces it to one-third to one-half its original strength; at 50° C. 50 per cent. is lost in five minutes. They have examined the rate of destruction at 55° and find that this goes on quite irregularly and is not in proportion to the length of time. The irregularity, however, presents a certain rhythm, *i.e.*, a period of greater destruction alternating with a period of less active destruction. Reudiger has studied the preservation of frozen complement and finds that at the expiration of one week whether the complement is made up of serum of a single guinea-pig or the pooled serum of several guinea-pigs the activity in the Wassermann is somewhat stronger than with fresh serum. At the end of two weeks the frozen complement gives results that are practically identical with the results obtained with fresh complement, but after two weeks the frozen complement gradually loses strength apparently more rapidly in mild weather than in very cold weather.

Variability of Complement.—Complementary activity varies considerably in different sera; in the same serum it may operate differently with amboceptors from several different species. The explanation of this difference of activity has led to a difference of opinion as to the exact nature of the complementary activity. Ehrlich and Morgenroth and the German school take the position that a given serum contains a considerable number of complements, whereas Bordet and his school take the point of view that the complement in any given serum is a unit, although they admit that complements in different sera may represent a somewhat different constitution.

Multiplicity of Complements.—Ehrlich and Morgenroth were able to show that the complementary activity of a serum could be divided by means of filtration in the following respect. They showed that complement for sensitized guinea-pig cells passes through the filter, whereas complement for sensitized rabbit cells remains in the filter. It has also been shown by thermal and chemical differentiation that

some complements are destroyed and others remain in the same serum. Weak acids and weak alkalis may differentiate complement similarly; it is stated that digestion by papain also serves so to differentiate. By injection of a complementary serum into foreign species, a so-called anti-complement is obtained which is said to act upon one of the complements of a serum and not upon others, irrespective of whether the complement of the antigenic serum or of some other serum be employed in the subsequent test. Practically all these experiments have been performed in such a way that the complement acts with normal amboceptors and the question at once arises as to whether or not the same phenomena would be observed with immune amboceptors. Even in the case of normal amboceptors, there is experimental contradiction of the original supposition of Ehrlich and Morgenroth. Neisser stated that anthrax bacilli deprive fresh rabbit serum of its bactericidal complement, but not of its hemolytic complement. Wilde showed that if a sufficient mass of anthrax bacilli were added to the fresh rabbit serum all the complement is used, so that further bactericidal action does not occur and no hemolytic action can be demonstrated. Similarly Bordet found that unsensitized red blood-corpuscles deprive a serum of only part of its complement but that cells strongly sensitized with hemolysin use up all the complement both bactericidal and hemolytic. He believes that the reason normal amboceptors do not utilize all the available complement is due to the fact that such normal amboceptors do not sufficiently sensitize the antigenic cells. Therefore, the complete sensitization of the cells will result in a complete utilization of complement. After the publication of these experiments, Ehrlich, who confirmed the results, explained the phenomenon as being due to a multiplicity of complements in the serum. In order to do so, he was obliged to alter the original conception of the amboceptor, so that instead of having a single cytophilic group it must contain several cytophilic groups. Therefore, the term was altered to polyceptor instead of amboceptor. The polyceptor was supposed to have one group with an especial affinity for a dominant complement and other receptors with affinities for the secondary complements. If the dominant complement is absorbed by the polyceptor, the secondary complements are also involved, but, on the other hand, if, as has been claimed, it is possible to obtain a serum with only secondary complements present, these may be absorbed without action upon the receptor for dominant complement. This explanation, however, rests entirely upon the Ehrlich conception of the amboceptor, and, inasmuch as this conception is not conclusively proven, it is not necessary to accept the idea that complements are multiple. This question, however, is not settled at the present time, and reference will be made to it again in connection with the phenomenon of complement fixation.

Complementoids.—The similarity in action and nature of complement and toxin was early recognized, and it was therefore attempted to determine whether or not complement could be broken up in the same way as toxin so as to form complementoids. If such were the case, it

should be possible to break up a complement so as to demonstrate a haptophore group and a zymophore or zymotoxic group. Thus, exposure to increased temperature might be so arranged as to destroy the zymophore group and leave the haptophore group intact. If this were true, the haptophore group or complementoid might be added to an antigen-amboceptor mixture and thus prevent any further action by a fresh whole complement. Experimentally, however, it was found that this, in the majority of instances, does not occur. Nevertheless, Ehrlich and Sachs found that if they mixed inactivated guinea-pig serum, normal inactivated dog serum and guinea-pig cells, hemolysis did not occur, even after the addition of fresh guinea-pig serum. They believed this to be the result of a blocking or plugging of the complementophile group of the dog amboceptor by the complementoid of the inactivated guinea-pig serum, thereby preventing the union when fresh active complement was added. Fuhrmann supported this statement and maintained that allowing the complement to stand for a period of three weeks was even more adapted to separation of the haptophore and zymophore group. Following this work, Muir and Browning conducted an extensive series of complicated experiments, which in the main tend to support the view of Ehrlich that complementoids actually exist. If they do exist, however, they are not uniformly demonstrable, and it may very well be that this is due to the difference in destructibility of the two groups being so slight that our methods of differentiating by means of heat and standing are not sufficiently exact.

Complement Fractions.—Further light was thrown on the possibility of fractioning complement by the experiments of Ferrata, who found that dialyzation of the serum resulted in the destruction of complementary activity. Dialyzation precipitates the so-called globulin fraction of the serum as contrasted with the albumin fraction which remains in solution. The precipitate may be dissolved in physiological saline and the portion in solution may be restored to its original salt concentration, thereby forming isotonic solutions of the two protein fractions. Ferrata found that neither of these components in the presence of an amboceptor was capable of producing hemolysis, but that if both were added, sufficiently soon after dialyzation, hemolysis would take place. Brand studied this phenomenon further and found that both fractions are equally thermolabile and because of activities which he discovered, named the fraction contained in the globulin precipitate "mid-piece" and that in the albumin "end-piece." If the amboceptor-cell mixture is treated first with mid-piece, no hemolysis occurs, but if end-piece is then added, hemolysis occurs as it would have in the original amount of complement. If the end-piece is first added and later the mid-piece, hemolysis will occur, but in very much smaller degree than if the entire complement had been used. Zinsser found, however, that when mid-piece and end-piece are mixed and then added to the sensitized cells, the hemolytic effect is reduced and is considerably less than if mid-piece is added before end-piece. It has been found that the mid-piece may enter into combination with the sensitized cells at 0° C., but the end-

piece combines only at considerably higher temperatures. It has also been found that the mid-piece of one animal species may be activated by the end-piece of another animal of the same or different species. Nevertheless, Ritz and Sachs have shown that the serum of an animal may possess a mid-piece for certain sensitized erythrocytes, but does not necessarily possess a corresponding end-piece. Marks has studied the quantitative relations of mid-piece and end-piece and has found that a ratio of 1-1 is not necessarily the optimum for hemolysis and that very often it is necessary to change the ratio; this change must be by increase of mid-piece, never by increase of end-piece. If the two are mixed before addition to the amboceptor-cell mixture, an excess of mid-piece inhibits hemolysis, but if the excess of mid-piece is added first followed by end-piece, hemolysis is complete. Brand and later Hecker found that if the globulin precipitate is preserved dry or in solution in distilled water it will retain activity for several days, but in physiological salt solution it loses its activity in three to four hours. This, however, does not indicate destruction of mid-piece in salt solution since it will combine with sensitized cells if added before end-piece. Marks holds that this phenomenon is due to the inhibition of hemolysis by excess of mid-piece and does not occur if proper proportions are maintained in the mixture. Swirski maintains that the complement fixation of the Wassermann test binds mid-piece but not end-piece. This has been investigated by Bronfenbrenner and Noguchi, who believe that the free end-piece in Wassermann tests differs from all other end-pieces in that it activates the complex which includes sheep cells but has no effect upon the cells of other animals. Bessemans has recently investigated again the question of thermostability of end-piece and mid-piece. He finds that there are important differences in certain of the sera he has examined, so that a general statement in regard to the heat resistance of these fractions is not justified.

Browning and Mackie have found that by various methods of fractioning the serum it is possible to divide complement into four fractions and that certain combinations consisting, as a rule, of at least three of these reproduce quantitatively the full hemolytic effect of the whole complement. This presents numerous intricate possibilities of experiment, but the important point is that such a demonstration makes the use of the terms mid-piece and end-piece no longer desirable.

Normal Hetero-hemolysins.—The preceding discussion has been concerned chiefly with complement and immune amboceptor. Historically much study had been directed toward the normal cytotoxic powers of blood serum before the immune amboceptors were recognized; Fodor, Nuttall, Nissen and Buchner had investigated the action of normal sera in dissolving bacteria. Buchner in 1893 pointed out a similar capacity of blood serum for dissolving animal cells, particularly erythrocytes. Ehrlich and Morgenroth took up the question as to whether or not the globulicidal activity of normal serum is due to an interaction of two substances similar to that in immune sera. They showed that normal dog serum is capable of dissolving guinea-pig erythrocytes, but

that it is inactivated by heating to 55° C. Reactivation by fresh dog serum was undesirable because of the normal amboceptor present. Therefore, they employed fresh guinea-pig serum in fairly large doses and reactivated the heated dog serum so that complete hemolysis occurred. Thus they demonstrated the double nature of the normal hemolysins and also that a complement may serve to hemolyze cells of the same species from which the complement is obtained. Other experiments have shown, however, that a complement operates less actively against homologous cells than against heterologous cells. Ehrlich and Morgenroth showed similar relationships by employing as the amboceptor normal calf serum and normal sheep serum, as well as similar hemolytic complexes with sheep and goat blood. They also showed that in a number of instances the amboceptor could be differentially absorbed by cells at 0° C., leaving complement free in the serum. Such absorption could not be accomplished with all normal hemolytic sera; in some the cells absorbed both amboceptor and complement, whereas in others no absorption whatever occurred. They interpreted the absorption of both bodies as due to the possession on the part of the amboceptor of equal avidity of both the cytophile and complementophile group, whereas failure of absorption was supposed to be due to a stronger affinity of complement for amboceptor than of cells for amboceptor. We record the fact without accepting the explanation, but it is important that in some instances normal hemolytic sera may fail to exhibit a separability of complement and amboceptor by means of differential absorption.

A normal serum may be hemolytic for cells of more than one species; this is true of goat serum, which is hemolytic for both guinea-pig and rabbit cells. In such cases it is possible to absorb one amboceptor, leave the other active in the serum and thus demonstrate multiplicity of specific amboceptors in a serum. Ehrlich and Morgenroth also maintained that in the case of goat blood there is a different complement for the two types of cells, but as has been indicated in discussing complement this possibility seems unlikely.

Proportions of Amboceptor and Complement in Normal Hemolysins.—Further difference between a normal and immune hemolytic serum lies in the different proportion of amboceptor and complement. In a normal hemolytic serum the amount of amboceptor present is small and the complement is usually present in at least sufficient quantity to saturate the amboceptor; it may be present in excess. In immune sera the amboceptor is increased enormously, whereas the complement is not altered in quantity. Therefore, such an immune serum may contain amboceptor in greater quantities than can be saturated by complement and for its full activity requires more complement than can be furnished in the immune animal's serum. This increase in amboceptor is out of all proportion to the amount of antigenic cells injected. Muir, for example, calculated that in immunizing a rabbit the total amount of ox blood injected was 30 c.c., and hemolytic amboceptor was produced sufficient to dissolve the erythrocytes in

6000 c.c. of ox blood. The practical bearing of the disproportion of complement to immune amboceptor lies in the use of bactericidal sera. It is easily conceivable that injections of such sera may meet in the injected animals' blood with an insufficient amount of complement for complete activation. Therefore, it may be advisable in such experiments or in therapeutic use of sera of this type to activate with a sufficient quantity of fresh complementary serum.

Normal Iso-hemolysins.—Attention has been called (page 99) to the phenomenon of iso-hemagglutination. Similarly iso-hemolysins can be demonstrated. For such a purpose the serum must be fresh and the corpuscles exposed to it at incubator temperature. It is probable that these hemolysins resemble other normal hemolysins. The groups correspond to the groups of iso-hemagglutinins. As in other experiments with hemolysins, agglutination appears and is followed by hemolysis. Agglutination inhibits hemolysis to a certain degree, as has been shown by Händel and by Karsner and Pearce. Kolmer, Trist and Flick have maintained in a recent study that there are two varieties of natural hemolysin and hemagglutinin in human sera. They find a thermolabile variety of these antibodies which is destroyed at 56° C. for thirty minutes and a less thermolabile or thermostable body which is destroyed at 62° C. for thirty minutes. Exposure at 56° C. removes the various iso-hemolysins but does not destroy the iso-hemagglutinins. Sands and West have found that if the immune sera are filtered (more particularly in 1–10 dilution) through perfectly clean Kitasato or Chamberland filters a large amount of the hemagglutinin is removed, with slight or no reduction of hemolytic activity. In fact, the hemolytic activity may be increased by the filtration and this may be explained as due to the removal of whatever inhibiting power on hemolysis hemagglutination may display.

Anti-amboceptors.—Inasmuch as the bodies which take part in hemolysis, the amboceptor and complement, are of protein nature, it is presumable that they might serve as antigenic substances. It should be possible to prepare anti-amboceptor and anti-complement. As previously mentioned, experiments have been reported which have been interpreted to indicate that it is possible to produce such immune anti-complements, but the evidence offered has not withstood criticism; at the present time it is extremely unlikely that true anti-complements have been demonstrated. Anti-amboceptors were first produced by Ehrlich and Morgenroth who injected as the antigen a hemolytic immune serum. If a hemolytic immune serum is injected in fairly large amounts into an animal for whose red cells the serum is specific, death results. By carefully-graded injections, however, it is possible so to immunize the animal that it becomes immune to the toxic effect of the serum. When so immunized the serum of this animal when added to a cell amboceptor mixture and incubated will prevent subsequent hemolysis on the addition of complement. Similarly anti-amboceptors may be produced by injecting serum containing amboceptors into other animals than those for which the serum is hemolytic. Ehrlich and Morgenroth

were of the opinion that such anti-amboceptors represented an excess of cell receptors formed during the course of immunization and were thus free in the serum to combine with the cytophilic group of the amboceptor. Bordet found, however, that it was not necessary to use a hemolytic immune serum as an antigen and demonstrated an anti-amboceptor by injecting normal rabbit serum into guinea-pigs. The anti-serum formed in the guinea-pig not only neutralized hemolytic amboceptor of rabbit serum but other amboceptors of rabbit serum as well, and therefore it could not be regarded as combining with such a specific receptor as the cytophilic group of the amboceptor. This work was confirmed by several investigators, including Ehrlich and Sachs, who agreed with Bordet that the anti-amboceptor does not combine with the cytophilic group but offered the new assumption that the combination is with the complementophilic group. Muir and his co-workers have studied anti-amboceptors extensively and find no good ground for accepting the later interpretation of Ehrlich and Morgenroth.

Muir offers an experiment as follows to illustrate the simple action of anti-amboceptor. Two tubes are marked A and B. Into each are placed one unit of cell suspension and three units hemolytic amboceptor (contained in rabbit serum), the mixture incubated and then washed and resuspended. To tube A is added 0.3 c.c. anti-amboceptor (prepared by injecting rabbit serum into guinea-pig), and to tube B is added 0.3 c.c. normal inactivated guinea-pig serum. The mixtures are again incubated and washed; to each tube is added one unit complement and the tubes are again incubated. Hemolysis is complete in tube B but is absent or much inhibited in tube A, thus demonstrating the inhibiting effect of the anti-amboceptor. Such an anti-amboceptor as is here illustrated will operate only against amboceptors contained in rabbit serum. Similar amboceptors contained in goat serum would not be affected by the anti-amboceptor prepared by injecting rabbit serum into guinea-pigs. If in the preceding experiment the supernatant fluid were examined after the first incubation it would be found that the amboceptor had been absorbed; a fact also illustrated by the hemolysis in tube B. Even were anti-amboceptor and amboceptor mixed and then added to cells the amboceptor would not be prevented from absorption. If the supernatant fluid were taken after the last incubation complement would be found free in tube A but not in the full original amount, as can be shown by careful titration. The anti-amboceptor keeps a certain amount but not all the complement from being utilized. The converse, however, cannot be demonstrated, that is to say, complement cannot be shown to inhibit in any way the union of amboceptor and anti-amboceptor. Intricate experiments demonstrate, however, that the union of amboceptor and anti-amboceptor is loose and a certain amount of dissociation may occur upon the addition of a normal serum homologous with the serum which contains the amboceptor.

Anti-complements.—As has been indicated above, it is improbable

that any so-called anti-complements operate differently from these anti-amboceptors. Numerous substances and physical conditions are anti-complementary in that they destroy or inhibit the action of complement. These have been pointed out in discussing the nature of complement and must be considered in all experiments which utilize complement. It has been suggested that in the interaction between amboceptor and anti-amboceptor a precipitate is formed which fixes complement and that if such were the case the complement should not be recoverable. Muir has shown that it is possible to recover complement, as we have pointed out above. Nevertheless, even such a form of fixation may permit of dissociation, and, as we shall show in discussing complement fixation, there is much evidence in favor of the view that the action of these antilysins is dependent upon the fixing properties of precipitates.

Physical Hemolysis.—Hemolysis is produced not only by the serum components discussed in the preceding paragraphs but also by chemical and physical agents, by bacterial products, by certain vegetable poisons and by venoms. Studies of these forms of hemolysis are of interest not only because of their intrinsic value but also because they serve to throw some light on serum hemolysis.

The necessity for using an isotonic salt solution for the preservation of erythrocytes is well known and equally well known is the fact that reduction of salt content of the menstruum beyond a certain point leads to solution of hemoglobin, which in distilled water is seen as complete hemolysis. This is not merely a question of solubility of hemoglobin for this substance is soluble in physiological salt solution to the same degree as in distilled water. For the same reason it is not a matter of simple osmosis of the hemoglobin. Although distilled water produces swelling of the cells before the solution of hemoglobin the rupture of the cell is of no especial importance for cells may be cut into pieces in physiological salt solution without hemolysis appearing. From experiments of Fischer it would appear that the hemoglobin is held in combination with the stroma by adsorption and that the action of the water causes a physical separation. By combining fibrin, a hydrophyllic solid colloid, with carmine, a hydrophobic colloid dye, Fischer was able to produce phenomena closely resembling hemolysis.

Fragility of Erythrocytes.—The corpuscles of different animals differ in the point to which reduction in salt concentration of the surrounding menstruum leads to hemolysis. This is spoken of as a difference in resistance to hypotonic salt solution or a difference in fragility. There may be a very slight difference in fragility of the corpuscles of different individuals of the same species and diseased conditions may lead to well-marked alterations. In man a simple secondary anemia may lead to a normal or reduced fragility, whereas pernicious anemia leads to increased fragility. Obstructive jaundice is accompanied by decreased fragility, whereas familial hemolytic jaundice shows increased fragility. In the anemia of animals following removal of the spleen there is a decrease of fragility or, in other words, an increase of resistance to hypotonic salt solutions and also to other hemolytic agents.

Hemolysis may be caused by other physical agents, such as freezing (particularly when followed by thawing), heat of 62° to 64° C. in the case of mammalian corpuscles or slightly less in the case of cold-blooded animals and, as Rous has shown, by shaking.

Chemical Hemolysis.—The influence of chemicals on hemolysis appears to be a factor of their permeation of the stroma. Wells states that there seem to be two types of permeating substances, one such as urea, which does not act in isotonic solutions of sodium chloride, and the other such as ammonium chloride, which acts either in isotonic or non-isotonic solutions. Hamburger, as quoted by Wells, states that erythrocytes in relation to organic substances are (a) impermeable for sugars, including cane sugar, dextrose, lactose, arabinose and mannose; (b) permeable for alcohols in inverse proportion to the number of hydroxyl groups they contain, also for aldehydes (except paraldehyde), ketones, ethers, esters, antipyrin, amides, urea, urethan, bile acids and their salts; (c) slightly permeable for neutral amino-acids, such as glycocoll and asparagin. In relation to inorganic substances, not including the salts of fixed alkalis, the erythrocytes are (a) "impermeable for the cations Ca, Sr, Ba, Mg, and (b) permeable for NH₄ ions, for free acids and alkalis." It will be noted that certain of the organic substances for which the cells are permeable are solvents of lipoids, particularly those lipoids of the stroma, cholesterol and lecithin, a phenomenon which will be referred to again in discussing venom hemolysis. Other chemical hemolysins include veratrin, digitalin, arseniuretted hydrogen (in the body but not in the test tube), nitrobenzol (important in denatured alcohol poisoning), nitrites, guaiacol, pyrogallol, aniline compounds, alcoholic extracts of tissues and products of tissue autolysis. Salt solution extracts of various organs are hemolytic and have been called organ hemolysins. These bodies resist boiling, do not act as antigens, hemolyze at 37° but not at 0° C., are not increased in activity by complement but are inhibited by the presence of serum. Noguchi has studied alcoholic tissue extracts extensively and finds them also hemolytic. He has come to the conclusion that the active elements in organ hemolysis are soaps.

Bacterial Hemolysins.—Bacteria may by their growth lead to sufficient acid or base formation in the media as to make the latter hemolytic. Of equal importance is the fact that certain bacteria may produce hemolytic bodies not of definitely acid or alkaline character, called bacterial hemotoxins. These substances include for the most part the products of pathogenic organisms, such as tetanolysin, staphylolysin, streptolysin, typholysin, vibriolysin (El Tor strain of cholera), anthrax-lysin and certain other less important forms. Certain saprophytes also are capable of producing lysins, as for example megatheriolysin, proteus-lysin and the lysin of bacillus Welchii and others of the gas gangrene group. An important bacterial hemotoxin is that of bacillus pyocyaneus. The exact nature of these substances is not known, but Burckhardt has shown that staphylolysin is dialyzable, thermolabile, ether soluble and does not give protein or biuret reactions.

The action on the cells is independent of complement, there is no combination at 0° C., but at 6° C. combination occurs, leading to hemolysis only at higher temperatures. It, therefore, seems likely that these bodies are similar to toxins with a special affinity for erythrocytes. The most favorable medium for developing these hemotoxins is broth, but individual organisms require special conditions in the broth for maximal production of hemotoxin. The development of the toxin follows fairly definite curves for the different organisms. For example, staphylolysin begins to appear on the third day, reaches a peak on the fifth day, drops on the sixth day, rises again on the eighth day, drops again and reaches a final maximum on the thirteenth day. Megatheriolysin reaches a maximum on the seventh day and almost disappears by the fifteenth day. De Kruif has recently shown that streptolysin reaches its maximum in a few hours and has almost disappeared by the end of twenty-four hours. The action is variable for different species of corpuscles; staphylolysin acts powerfully on horse, sheep and other bloods but only slightly on human and goat blood, whereas megatheriolysin acts powerfully on human blood but not at all on horse blood. Nakayama has studied the streptolysin and finds that it is filterable. He also passed the organisms through two species of animals and finds that after animal passage the streptolysin is more actively lytic for the corpuscles of the species which last harbored the organisms. Streptolysin unites with the corpuscles in the course of hemolysis, but the filtrate, after absorption of lysin, remains toxic for mice. Many of the bacterial hemotoxins are thermolabile, being destroyed at 60° to 65° C., but others are resistant to temperatures as high as 100° C. The bacterial hemotoxins are active *in vivo* as well as *in vitro* and are capable of producing severe anemias and even death. An intravenous dose of 2.0 c.c. of a ten-day-old filtered broth culture of staphylococcus produces in the rabbit marked reduction in hemoglobin and the number of erythrocytes and may cause death in six or seven days. It is probable that the secondary anemias which often follow attacks of acute infectious disease may be dependent upon bacterial hemotoxins. Ford, Lawrence and Williams have found that cultivation of the bacillus *Welchii* in milk leads to the formation of bacterial hemolysins, thus disproving the opinion previously held that the hemolysis in gas bacillus infection was due to the formation of lactic or butyric acid. The hemolysin described by Ford and Lawrence is relatively stable, not being destroyed until a temperature of 62° or 63° C. has been reached. It has other characters of true toxin in that it is digested by pepsin and hydrochloric acid as well as by pancreatin. It is precipitated by ethyl alcohol. It is antigenic, and these investigators found that they could, by immunization, produce an anti-hemolysin or anti-hemotoxin in titers of 1-1000, 1-1250.

Vegetable Hemolysins.—Among the hemolytic substances of vegetable origin are to be included those already discussed as phyto-toxins, namely ricin, abrin, crotin, robin, phallin. Crotin and phallin are more markedly hemolytic than the others, which are rather hemag-

glutinative than hemolytic. The phytotoxins resemble some of the bacterial hemotoxins in that they may serve as antigens for the production of antitoxins but differ in that, as a rule, they are thermostable. Both groups act according to the law of multiple proportions. Of considerable importance from the experimental point of view are the saponins "a closely related group of glucosides found in at least forty-six different families of plants" (Wells). They are thermostable, do not act as antigens, have a fairly definite chemical composition and are in these particulars to be separated from true toxins. They operate injuriously not only upon the erythrocytes but also on other body cells, especially those of the central nervous system. Cholesterol and lecithin both combine with saponin, the former in such a way as to prevent hemolysis. Therefore, it is assumed that the hemolytic action of saponin is dependent upon its action on the stroma lipoids. Normal serum is anti-hemolytic for some of the saponins, a property which may be slightly increased by careful immunization; Kobert believes this increase to be due to an increase of blood cholesterol.

Venom Hemolysins.—The venom hemolysins or hemotoxins are found in different amounts in all venoms, and the phenomenon of venom lysis is of considerable importance not only because of its scientific interest but also because of its employment in certain clinical tests. The venoms possess not only lytic but also hemagglutinative properties, the two usually being present in inverse ratio. Flexner and Noguchi demonstrated that the lysis of venoms requires activation by some substance which exercises a complementary power. They found that cobra hemotoxin dissolves the red corpuscles of certain animals (ox, sheep and goat) only in the presence of serum, but that it may dissolve other erythrocytes (dog, guinea-pig, man, rabbit, horse) in the absence of serum. This difference is probably due to a content of activator in the latter cells, which activator must be furnished by serum for the lysis of the former cells. Kyes found that he could extract an activator from those cells which do not require serum for venom lysis but was unable to do so in the case of those cells which require serum activation. This activating substance was found to be ether soluble. Kyes subsequently found that lecithin can activate venoms and assumed that this lipid constituted the bulk of the activating substance. The substance in serum is usually active only after the serum has been heated, but with some sera heating is not necessary. Kyes and Sachs believed this to be due to differences in the nature of the lecithin union in the serum. Kyes mixed cobra poison with a chloroform solution of lecithin and obtained a substance which he named cobra lecithid capable of activating cobra venom. Von Dungern and Coca, upon investigating the subject, came to the conclusion that the cobra venom contains a ferment capable of splitting the lecithin so as to yield certain substances such as oleic acid and that the resulting hemolysis is due to the activity of these secondary substances. Noguchi holds that although lecithin exists in the stroma of red blood-cells it is not present in a form available for venom activation and that the

degree of susceptibility to hemolysis depends upon the amount of such ether soluble activators as fatty acids (particularly oleic acid) and their soluble soaps. He regards fatty acids, neutral fats and soluble soaps as endocellular complement and assumes certain similarities with serum complement. Certain soap serum mixtures were found to be capable of completing an amboceptor cell mixture but numerous objections have been interposed against both the fact and the interpretation so that at the present time there is no good ground for believing that the activator of cobra lysin is a true complement or that Noguchi's soap mixtures are comparable to serum complement. If the activator cannot be regarded as complement, the venom lysin cannot be looked upon as an amboceptor, for it shows no specificity and does not require serum complement for activation. Kyes in a recent publication gives what may be regarded as the modern view in regard to venom lysis as follows:

"1. There is present in all venoms a hemolysin existing as one of a number of distinct toxins.

"2. This hemotoxin effects hemolysis only in conjunction with a so-called complementing substance which, however, may be found within the erythrocytes.

"3. The reaction between the hemotoxin and lecithin is essentially a chemical reaction resulting in the formation of a complete lysin.

"4. This complete lysin is a true toxin in that it stimulates the production of a specific antibody."

Although the experiments of Zunz and György are not to be regarded as indicating that they have found other activating substances for cobra venom hemolysis, nevertheless, they have determined that hemolytic activity of cobra venom is increased by certain compounds of protein destruction, including certain albumoses and amino-acids.

The hemolytic property of cobra venom has served as a basis for proposed clinical tests. Calmette noted that in tuberculosis the blood contains more than the usual amount of lecithin and that small amounts of serum of such patients served to activate cobra venom lysin. The test is not positive in more than 78 per cent. of tuberculous patients and, furthermore, is by no means specific. Similar increases of lipid content of serum have been found in certain diseases of the central nervous system, a fact leading to the Much and Holzmänn psycho-reaction, which also is not specific. Weil has maintained that in syphilis the corpuscles are more resistant to venom hemolysis than is normal, except in the earlier stages where the corpuscles are said to be hypersensitive. As time has passed the suggested clinical tests have not come into general use largely because of lack of specificity. Undoubtedly the blood exhibits alterations in lipid content at different times in various diseases, and in all probability there is a parallel alteration in its power to activate venom lysin, but no one disease shows this change exclusively or even constantly.

Cytotoxins. Specificity.—As erythrocytes may act as antigenic substance, so may other body cells. The antibodies produced by injec-

tion of these latter cells were called cytotoxins by Metchnikoff, and the name has been retained in spite of the fact that the immune bodies are not toxins in the strict sense of the word but are amboceptors similar to the hemolytic amboceptors. It was thought that cytotoxins might be strictly specific for the antigenic cells of different organs within the same species, but more thorough investigation has shown that such "organ specificity" is not demonstrable. Hemolysins, for example, are lytic for other body cells, such as liver and kidney, provided these cells are of the same species. Hepatolysins and nephrolysins are also active as hemolysins within the species. In other words, these antibodies are species specific but not organ specific. It may be true that a cytotoxin acts more especially on its antigenic organ cells than upon other cells, as is maintained by Pearce for the nephrolysins, but the action is not exclusively upon the antigenic cells. In an extensive study Pearce, Karsner and Eisenbrey were unable to demonstrate any strict "organ specificity" by means of cytolysis, precipitation, agglutination or the anaphylaxis reaction. In this work the organs were washed by perfusion with large amounts of salt solution and thus prepared for injection. Bell has pointed out that even the most careful perfusion will not entirely remove the blood from organs and therefore a certain amount of blood must be injected with the other cellular antigen. Nevertheless, the early work of Landsteiner, Metchnikoff and of Moxter showed that spermatozoa which can be obtained free from blood may lead to a spermatolysin which also acts as a hemolysin. The amount of blood injected with carefully-washed organs is so small that it can have but little antigenic power, too little to be consistent with the well-marked hemolytic power of the cytotoxic sera. Recently, however, Wilson and Oliver have absorbed the hemolysin from cytotoxic sera by means of erythrocytes and maintain that there is a very definite organ specific cytotoxin contained in nephrolytic serum prepared by immunizing with kidney substance. This specific nephrolysin can be removed by absorption with kidney substance but not with other organs. If these studies are extended and confirmed, much new light may be thrown on the subject of organ specificity.

In spite of the apparent lack of strict organ specificity, the cytotoxins of certain types of cells deserve mention, namely those resulting from the injection of leucocytes and of crystalline lens. Following a brief communication by Delezenne concerning leucotoxins, Metchnikoff studied the matter by injecting guinea-pigs with material from the mesenteric lymph-nodes and from the bone marrow of rabbits. The resulting immune serum was highly toxic for guinea-pigs, but if given in sufficiently small doses produced first a marked leucopenia, followed in several days by a leucocytosis. This was confirmed by others who used for injection also leucocyte emulsion, and although species specificity was strict, the cellular specificity was not. Lucatello and Malon were able to obtain a serum by the use of leucocytes from cases of leukemia and treated a series of cases with this serum. The leucocytes were reduced in number and the spleen diminished in size, but

there was no permanent improvement. The lack of cellular specificity in such sera is an *a priori* argument against their use.

Lens Cytotoxin.—The injection of crystalline lens leads to the formation of a cytolsin which is organ specific but not species specific, similar to the production of precipitins by lens protein. Such a cytotoxin prepared by the use of the crystalline lens of the dog is specific for all mammals, birds and fish and will not act upon other cells from these animals. The fact that the injection of lens into animals of the same species or even into the same individual leads to the production of isocytotoxins and autocytotoxins led Römer to build up a theory concerning the origin of cataract. He suggested that the constant absorption of lens protein from the normal process of tissue wear and tear leads to the development of an isocytotoxin which in later life produces the degeneration of the lens seen in cataract. If such were the case cataract should be a much more frequent complication of age than it is and the lens should be a soft pulpy organ as the result of cytolysis. Furthermore, complement is not available in the fluids of the eye and the cytolytic amboceptor is not to be completed in that position. Other theories as to the etiology of cataract are so much more logical that Römer's hypothesis has been practically abandoned.

Aside from the foregoing example of isocytotoxin and autocytotoxin formation, there are no well-determined illustrations of this phenomenon except for the demonstration by Metchnikoff of isospermatotoxins.

Bacteriolysins.—The death and solution of bacteria in the processes of resistance to disease may be accomplished by the activity of phagocytic body cells or by virtue of properties of the blood and body fluids similar in every way to those properties which lead to hemolysis. Indeed, the discovery of bacteriolysis antedated that of hemolysis even to the point of understanding the essentials of its mechanism. Nuttall in 1888 demonstrated that fresh normal defibrinated blood has the power of killing bacteria. He set up a series of tubes, each containing the same amount (0.5 to 1.0 c.c. defibrinated blood) and added to each a small platinum loopful of material from the spleen of a mouse previously inoculated with anthrax. These tubes were incubated and at different time intervals gelatin plates were made from the tubes and a control made from the splenic material. This showed that as incubation proceeded the bactericidal activity of the blood became apparent. Buchner confirmed this fact in 1889 with a slightly different method, whereby a larger amount of blood and bacteria were mixed in one container and incubated and small standard amounts withdrawn by pipette and plated. It was found that if the blood were heated or allowed to stand, its bactericidal power was lost and Buchner named the thermolabile element alexin. He believed it to be of the nature of a ferment, suggested that it might originate in body cells, possibly leucocytes, and recognized the fact that it is not specific.

The Pfeiffer Phenomenon.—The next important advance appeared in the studies of Pfeiffer and his co-workers, who, in 1893, 1894 and subsequently, published the details of what we now speak of as the



FIG. 15.—Stages in lysis of cholera vibrios, showing the reduction to large coccoid forms before final solution. (Modified from Pfeiffer and Friedberger, *Lehrbuch der Mikrobiologie*, Jena, 1919).



Pfeiffer phenomenon. These discoveries were incident to the investigation of immunity to cholera spirilla. The method is essentially that of studying the changes taking place in the spirilla following intraperitoneal injection in guinea-pigs. If the guinea-pig had survived preceding inoculations and had thereby developed immunity the injection of organisms was followed by loss of their motility, transformation into oval translucent granules and finally disappearance of the bacteria with complete recovery of the animal. If the spirilla were of only low degree of virulence the same phenomenon could be observed in a normal animal, but if the animal were highly immune it could survive doses of virulent organisms much greater than those fatal for normal guinea-pigs. It was found that the simultaneous intraperitoneal injection of serum from an immune pig and of spirilla into a normal pig served to protect the animal and that this protection could be conferred as well by heated as by non-heated immune serum. The mechanism in all cases was the same and not dependent upon phagocytic activity. Furthermore, the protection was found to be specific. Pfeiffer was unable to demonstrate the phenomenon *in vitro* (hanging drop preparation) and therefore assumed that some substance provided by the peritoneal endothelium served to activate the bacteriolytic process.

In the demonstration of the Pfeiffer phenomenon it is necessary to have a series of fairly young guinea-pigs of about 200 grams in weight and a culture of cholera spirilla whose virulence is well established, because the virulence of the organisms plays quite as important a part as their number. The immune serum may be produced in the rabbit, goat or other animal by repeated inoculation with the organisms. The organisms may be injected in measured volumes of broth cultures or of saline suspensions of agar cultures; they may also be measured by weight by the use of a standard platinum loop which takes up approximately 0.002 gm. organisms. The immune serum is diluted as indicated in the following protocol and the bacteria and serum are injected simultaneously. Peritoneal fluid is withdrawn at intervals of 10, 20, 30, 45, 60 minutes, the intervals being altered as circumstances indicate. The withdrawal is by means of drawn out capillary pipettes introduced into the belly cavity through a small incision in the skin. The material may be examined in a hanging drop or may be spread and stained by the ordinary bacterial dyes. A protocol from Pfeiffer's own work follows:

PFEIFFER PHENOMENON

Weight of guinea-pig in grams	Dose of spirilla in grams	Dose of immune serum in c.c.	Result	Examination of peritoneal fluid
320	0.002	0.05	Lives	After 15 minutes, only granules present.
240	0.002	0.02	Lives	After 20 minutes, only granules present.
200	0.002	0.006	Lives	Sterile after 35 minutes.
220	0.002	0.003	Lives	After 25 minutes, numerous granules, isolated, non-motile spirilla. After 1 hour practically sterile.
220	0.002	0.001	Died during night	After 25 and 50 minutes, numerous granules but also numerous active spirilla. After 100 minutes, only active spirilla.

PFEIFFER PHENOMENON—(Continued)

Weight of guinea-pig in grams	Dose of spirilla in grams	Dose of immune serum in c.c.	Result	Examination of peritoneal fluid
230	0.002	0.0005	Died during night	After 25 minutes, a few granules, numerous active spirilla. Progressive increase of spirilla.
200	0.002	0.2 c.c. normal guinea-pig serum as control	Died during night	After 25 minutes, few granules, numerous active spirilla. Autopsy after several hours showed pus on the liver, numerous spirilla mostly free in exudate, with granules both free and within cells.

In the foregoing experiment it is seen that 0.003 c.c. immune serum serves to protect a guinea-pig of about 200 grams from an otherwise fatal dose of cholera spirilla. Pfeiffer used this method to titrate bacteriolytic sera and in this case would have indicated the serum as containing in each cubic centimeter 333 immune units.

Bacteriolysis in Vitro.—Further study of the phenomenon more particularly by Metchnikoff and by Bordet led to the discovery that the process may be demonstrated *in vitro*, in spite of Pfeiffer's failure to do so. Metchnikoff was able to produce lysis of spirilla in hanging drop preparations by adding to the mixture of spirilla and immune serum an extract of leucocytes, thus offering evidence in favor of the influence of leucocytes in destruction of bacteria. Bordet demonstrated that although the activity of the immune serum is destroyed by heat of 50° C. to 60° C., the serum may be rendered active again by the addition of a small amount of fresh serum, an amount of fresh serum in itself incapable of producing bacteriolysis. He found that the specificity of the immune serum resides in a substance which he later named the sensitizer (the Ehrlich amboceptor). The alexin of Buchner (complement) was found to exhibit no specificity and was not increased by immunization. In the course of these studies Bordet found that the corpuscles in the fresh normal guinea-pig serum were agglutinated by the immune goat serum and that the spirilla were often likewise agglutinated. Suspecting that if both blood-cells and bacteria are agglutinable, the blood-cells might be the subjects of lysis as well as are bacteria, Bordet was led to the discovery of the phenomenon of hemolysis. The studies of Toitsu, Matsunami and Kolmer would indicate that all bacteriolysis is not necessarily dependent upon the activity of complement, for they found that anti-meningitis sera which were freed from complement possessed bactericidal properties. Ecker has made similar observations in regard to a serum specifically bacteriolytic for the diphtheria bacillus. Nevertheless, Ecker found that the addition of complement increases the bacteriolytic action of this serum.

The Pfeiffer phenomenon was found applicable to bacteria other than the cholera spirilla, including particularly the typhoid bacillus, paratyphoid, dysentery and colon bacillus. With these organisms the phenomenon proceeds more slowly than with cholera spirilla. Were

no simpler means available, the Pfeiffer phenomenon might well serve as a laboratory method of identifying cultures of the bacteria.

Wright's Method for Bacteriolysis.—In the course of subsequent studies, other methods of investigation of bacteriolysis have been devised, those of Wright, of Neisser and Wechsberg and of Buxton deserving especial mention. Wright exercised his usual ingenuity in attacking this problem and devised two methods, one by dilution of serum and the other by dilution of the culture of organisms. For the collection of serum he used the Wright pipette such as is employed for determining opsonic content of serum. The serum was diluted with different amounts of bouillon. The culture was mixed with melted gelatine and to measured amounts of this mixture was added the proper amount of serum dilution. The final mixtures were incubated in capillary pipettes for two to three days at 22° C., then placed under low magnification of the microscope and the number of colonies in the pipettes determined. In the second method the culture was diluted in varying amounts of broth by means of a specially constructed capillary pipette and the suspension blown into a watch glass. The culture dilutions were mixed with a standard amount of serum and incubated in special pipettes. If the serum was insufficient to kill all the organisms, there was bacterial growth, and the medium became cloudy. Having, by previous plating, determined the number of organisms in a given bulk of broth culture, it was possible to determine how many organisms could be killed by the standard amount of serum. The outlines of these methods are given because of the ingenuity displayed and the exact information gained, although at the present time they are not extensively employed.

The Neisser-Wechsberg Phenomenon.—The Neisser and Wechsberg method was described almost contemporaneously with that of Wright. They mixed inactivated serum dilutions in test tubes with either broth cultures or salt solution suspensions of organisms, added complement and incubated. Definite amounts of these mixtures were added to melted solid culture media, such as agar, and plates poured. After incubation of the plates, the colonies were counted and the bacteriolytic activity of the serum thus determined. A protocol taken from the studies of Neisser and Wechsberg will serve to illustrate the method.

NEISSER-WECHSBERG PHENOMENON				
Tubes	Amount of culture	Inactivated immune serum	Fresh guinea-pig serum	Number of colonies on plates
1	1/5000 c.c. of a 24-hour broth culture of vibrio Metchnikovi	1.0 c.c.	0.3 c.c.	Many thousands
2	"	0.5 c.c.	0.3 c.c.	Many thousands
3	"	0.25 c.c.	0.3 c.c.	Many thousands
4	"	0.1 c.c.	0.3 c.c.	Few hundred
5	"	0.05 c.c.	0.3 c.c.	About 100
6	"	0.025 c.c.	0.3 c.c.	About 50
7	"	0.01 c.c.	0.3 c.c.	None
8	"	0.005 c.c.	0.3 c.c.	None
9	"	0.0025 c.c.	0.3 c.c.	About 100
10	"	0.001 c.c.	0.3 c.c.	Many thousands
11	"	0.0005 c.c.	0.3 c.c.	Many thousands

NEISSER-WECHSBERG PHENOMENON (*Continued*)

Controls	Amount of culture	Inactivated immune serum	Fresh guinea-pig serum	Number of colonies on plates
1	1/5000 c.c.	Many thousands
2	1/5000 c.c.	0.01 c.c.	...	Many thousands
3	0.01 c.c.	...	None
4	1/5000 c.c.	...	0.3 c.c.	Many thousands
5	1.0 c.c.	None

The broth culture is so diluted that 0.5 c.c. are added to each tube. All tubes are made up to constant volume with 0.85 per cent. salt solution. Incubation is for three hours at 37° C., after which five drops from each tube are added to a tube of melted agar for plating.

The Neisser-Wechsberg method not only presents a means of working with bactericidal sera but also demonstrates both the necessity for the presence of complement to complete the bactericidal amboceptor and the appearance of inhibition zones in the stronger concentrations of the immune serum. Neisser and Wechsberg interpreted the inhibition zone as illustrating what they called "complement deviation," a term frequently used incorrectly as synonymous with complement fixation. They believed that if an excess of amboceptor units is present, a certain number of these units will combine with the available complement units, thus leaving a number of amboceptor units unsaturated with complement. The amboceptor is present in amounts too large to be entirely absorbed by the antigenic bacteria and therefore it is assumed that a certain number of the free amboceptor units combine with a number of complement units, thus preventing a sufficient amount of complement to combine with the amboceptor units already absorbed by the bacteria for the process of bacteriolysis. In tubes four to nine of the preceding protocol the amboceptor units and bacteria are closely enough balanced to ensure complete absorption of amboceptor and thus permit of full action of complement; there being no free amboceptor, there is no "deviation" of complement. Except for the possible evidence afforded by the Ehrlich and Sachs experiment (see page 125) there is no other experimental evidence supporting the view that free amboceptor may enter into combination with complement. Gay has suggested that the inhibition may be due to precipitation by the interaction of the immune serum and bacterial protein which may have gone into solution, the precipitate operating to fix complement and prevent its combination with bacteriolytic amboceptor. Whilst precipitation may undoubtedly be of significance in this connection, we are of the opinion that the resemblance to colloidal reactions as described in connection with precipitation and agglutination, wherein excess of one colloid may prevent the occurrence of precipitation or flocculation, offers an equally satisfactory explanation for the Neisser-Wechsberg phenomenon and that we are therefore justified in regarding the reaction as illustrating "inhibition zones" where the concentration of amboceptor is great. The failure of bacteriolysis in tubes eleven and twelve is due, of course, to insufficient amount of amboceptor. The control tubes show that neither amboceptor nor complement alone is capable of producing bacteriolysis.

Buxton's Method for Bacteriolysis.—Buxton determined that

active immune serum shows the same inhibition zones and also simplified the method. By allowing the original tubes to incubate twenty-four hours at 37° C., the degree of clouding of the medium by bacterial growth gives an excellent indication of the degree of bacteriolysis. He found that normal rabbit serum shows bacteriolytic powers in strong concentration, gradually diminishing as dilution proceeds. Thus the low titer normal amboceptor fails to show inhibition zones, as is true of low titer agglutinins and precipitins. A protocol from Buxton's work shows the difference in activity of normal serum and immune serum as well as the correspondence between the results of plating and observation of original tubes.

BUXTON EXPERIMENT

Dilution of sera	Count of colonies on plates		Observation of original tubes	
	Normal serum	Immune serum	Normal serum	Immune serum
1	0	Many thousand	Clear	Cloudy
1-2	0	Many thousand	Clear	Cloudy
1-5	2	Many thousand	Clear	Clear
1-20	2500	4-5000	Cloudy	Clear
1-40	Many thousand	4-5000	Cloudy	Clear
1-80	Many thousand	Many thousand	Cloudy	Cloudy
1-100	Many thousand	Many thousand	Cloudy	Cloudy

Teague and McWilliams have confirmed the work of Buxton and others showing that normal rabbit serum is capable of killing large numbers of typhoid and paratyphoid bacilli, but that the sera of rabbits highly immunized against these organisms do not kill these bacilli. These investigators have emphasized further that the normal bacteriolytic activity of rabbit serum for typhoid and paratyphoid bacilli is not materially altered by immunization. In human typhoid fever the blood serum normally shows bacteriolytic activity, but in spite of this bacteria multiply in the tissues apparently because the lymph does not possess bacteriolytic powers. Stone more recently made similar observations but found further that fresh immune typhoid serum *in vivo* has apparently a high bactericidal power, while fresh normal serum *in vivo* has no protective power. Typhoid bacilli disappear more quickly from the organs of immune animals than from normal animals, but macerated organs from immune animals, cut sections, or their extracts are not bactericidal even on the addition of fresh immune serum. This work indicates that the destruction of typhoid bacilli in the immune animal is due to some interaction between the tissue cells and plasma *in vivo* or other unknown factor.

The Bioscopic Method for Bacteriolysis.—Neisser and Wechsberg also devised the so-called bioscopic method of studying bacteriolysis. They took advantage of the fact that living cells possess the power of converting methylene blue into its colorless leucobase. By careful adjustment of the number of bacteria it was possible to mix the various agents together, add a very dilute alcoholic solution of methylene blue, cover with paraffin and incubate. The degree of decolorization indicates the relative amount of bacterial growth.

Summary of Cytolysis.—In summary it may be said that the phe-

nomenon of cytolysis represents a general biological phenomenon applicable to vegetable cells, exemplified by bacteria, and also to a wide variety of animal cells. In both kingdoms there is a marked species specificity exhibiting, as do other immune processes, the phenomenon of group reactions. In so far as bacteriolysis is concerned, inhibition zones appear, apparently similar to the inhibition zones of precipitation and agglutination. Two bodies interact to produce cytolysis, a thermostable body, the amboceptor or sensitizer, which may be increased by immunization, and a thermolabile body, the complement or alexin, which does not react to immunization. The amboceptor appears to act by preparing the antigenic cells for the lytic action of the complement rather than by furnishing a two-armed link between cell and complement. The reaction takes place more nearly according to the physical chemical laws of colloidal reactions than the simpler laws of reactions between inorganic chemicals. The protection afforded an animal which possesses bacteriolytic immune bodies is obvious, and the rôle these bodies play in natural and acquired immunity to disease must be of great importance.

CHAPTER VII

CELLULAR RESISTANCE

PHAGOCYTOSIS.

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INGESTION.

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PHAGOCYTOSIS

Introduction.—Metchnikoff has defined the phagocyte as a cell capable of ingesting foreign bodies. Similarly the process of phagocytosis can be referred to as the process of ingestion of foreign bodies by a cell. In the study of unicellular organisms and of certain lower forms of multicellular organisms it has been found that the process of phagocytosis is an important means of obtaining nutrition. That such a simple process could have any bearing on the resistance of vertebrates to disease was not pointed out for many years. The very earliest study of bacteriology and immunity led to the knowledge of the fact that the injection of bacteria into animals led, under favorable conditions, to the disappearance of these bacteria. The investigation of the cause of this disappearance resulted first in the belief that it was due to solution of the bacteria by body fluids, more especially the blood. Certain early investigators had noticed that following such injections of bacteria the bacteria might appear within tissue cells, but Panum

was the first to interpret the phenomenon in an immunological sense. He pointed out that the penetration of bacteria into living cells, as previously maintained by Birch-Hirschfeld, probably had much to do with the disappearance of bacteria following injection. Subsequently, it was shown that bacteria do not penetrate into cells but rather are taken up by the cells. This work of Panum did not lead to any direct result in the study of immunology, for Metchnikoff in his early work on the subject was ignorant of it. Roser had also stated that according to his opinion the immunity of healthy animals and plants against infectious organisms rests upon (*a*) the relative salt content of their fluids and (*b*) the capacity of their contractile cells to take up the invading organisms. Roser, however, did not support this statement in later studies, and again Metchnikoff was ignorant of this work when he took up his great work on phagocytosis. Metchnikoff had studied extensively the nutrition of certain of the lower forms of animal and vegetable life and also their defenses against the invasion of harmful parasites. From this work he was led to the conclusion that the defense of higher animals depends in great part upon the phenomenon of phagocytosis. This statement was magnified into a conflict between the so-called cellular theories of immunity and the humoral theories of immunity. At the present time, however, such a conflict does not exist because the two theories of immunity are in perfect harmony with one another, and it is known that they are dependently interrelated.

The process of phagocytosis involves three steps; first, the approach of the cell and the material to be taken up; second, the ingestion of the material, and, third, the destruction of such material as may be dissolved by the digestive fluids of the cell. The problem of the approach of the cell and material to be ingested is one fundamentally of irritability. The irritability of living tissue is in response to certain stimuli and such stimuli include chemical, thermal, osmotic, photic, mechanical and other physical agents. In the early studies of the physiology of stimulation the response of a cell to a stimulus was believed to be governed by the Weber-Fechner law, which states that the intensity of sensation varies with the logarithm of the intensity of the stimulus or, in other words, as the stimulus increases by geometric progression the response increases by arithmetical progression. This law has been found by further study to be untenable, for it has been shown that logarithmic functions are not applicable to very strong stimuli. In phagocytosis chemical stimuli are the most important, and the response to such stimuli is referred to as chemotaxis.

Mutual Approach (Chemotaxis).—Chemotaxis may be positive or negative, according to whether it attracts the two bodies or repels them. Such attraction or repulsion does not depend essentially on the acidity or alkalinity of the medium but does depend in certain measure upon its concentration. Not only does variability of concentration play a part, but the adaptability of the cell itself is of importance; for example, myxomycetes plasmodia exhibit negative chemotaxis in the presence of sugar in certain concentrations, but after the organism becomes

accustomed to the presence of the sugar a positive chemotaxis appears. The lower animal and vegetable cells exhibit a certain amount of selection in the material which they take up, and the leucocytes of higher organisms may in a similar manner exhibit selectiveness. According to our present-day physical conception of the activity of living protoplasm, this selectiveness would depend in all probability upon varying sensibility to chemotactic influences or variation in the intensity of the chemotactic stimuli.

Ingestion of Foreign Body.—The actual ingestion of the foreign body depends upon the motility of the cell protoplasm, and this motility, of course, is a function of the irritability of the protoplasm. Such motility determines the ameboid movement of the cell to the material to be ingested. Having approximated itself to the foreign material, the cell throws out pseudopodia in such a fashion as to encircle the foreign body; as opposite pseudopodia meet the cell resumes in so far as possible its normal form and the material is enclosed in the cell protoplasm. These two stages in the process of phagocytosis have been reduplicated in experiments with non-living material.

Digestion.—The third stage of phagocytosis is the digestion of the foreign material. Such digestion is accomplished by secretions which are poured out by the cell protoplasm so as to constitute a small area of fluid about the ingested particle. By staining with dyes which show the acid reaction it has been found that although the cell protoplasm does not show acidity the fluid within the digestive vacuole is definitely acid in character. Attempts to extract this digestive fluid from protozoa have not been highly successful, but Mouton was able to extract from a symbiotic culture of amebæ and colon bacilli an enzyme which is feebly proteolytic. This enzyme is capable of digesting colon bacilli which have been killed but does not act upon living colon bacilli. The intracellular digestion of these particles depends upon their solubility by the digestive fluids. The cell may take up insoluble particles, in which case the particles remain within the cells or are extruded with the excreta of the cells.

Types of Phagocytic Cells.—It is incorrect to think of leucocytes as identical with phagocytes, for numerous other body cells show this capacity, including the eosinophiles, the endothelial cells, the pulp cells of spleen and lymph-nodes, connective tissue cells, including bone cells, striated muscle cells and giant cells. It is probable that the lymphocytes and the mast cells exhibit no phagocytic activity. Metchnikoff divided phagocytes into microphages and macrophages. The microphages include particularly the neutrophilic leucocytes and the eosinophilic leucocytes, the important phagocytes of the circulating blood. The macrophages include the other cells mentioned above, the most important being the endothelial cells. It is perfectly true that the endothelial cell circulates in the blood, but apparently its most important activity is in the tissues and body spaces. The microphages are the more sensitive of the two groups and react not only to chemical stimuli but also to tactile and physical influences.

Functions of Phagocytosis.—Phagocytosis plays an important part in the entire life of the mammalia, even though the differentiation of many cells excludes them from this function. The destruction of erythrocytes in spleen, liver and bone marrow is in part due to phagocytosis. Involution of the uterus after pregnancy, involution of senile ovaries, decrease in substance of the brain and other organs in old age are due to phagocytosis. Metchnikoff has laid considerable stress upon the activity of phagocytes in the atrophic processes of old age. Rindfleisch claims to have demonstrated that phagocytes are active in the breaking down and removal of gouty deposits in and about joints. The fixed tissue phagocytes which play a part in the physiological destruction of red blood-corpuscles have been designated by Kyes as hemophages. In various animal species the blood destruction accomplished by the hemophages may be carried on predominately in one organ or another, the site of destruction, however, being constant for a given species under normal conditions. Pearce and his co-workers have shown that extensive blood destruction increases the phagocytic activity in the spleen and liver. They have also shown, as has been confirmed by us, that the removal of the spleen results in an assumption of hemophagocytic activity by the endothelial cells of the lymph-nodes. Cary has demonstrated that the injection of foreign red blood-corpuscles markedly increases the hemophagocytic activity of the recipient, not only in the spleen, which normally plays the important part in destruction of the red cells, but also in other organs. The resistance of the organism to foreign bodies, either living or inert, is partly the result of the same process.

Under abnormal circumstances the removal of tissue and cell detritus is due in part to phagocytosis. In the inflammatory reaction following the introduction of foreign bodies, especially infective bacteria, phagocytosis is the first line and most important defensive mechanism against invasion. Dusts inhaled into the lungs are taken up by mononuclear phagocytes or macrophages and conveyed to neighboring lymphatics and lymph-nodes, thus preventing accumulation on the respiratory membrane. In inflammation the circulation is slowed in the small vessels of the neighborhood, thus permitting the accumulation of leucocytes on the inner wall of the vessels. They then migrate through the vessel walls by ameboid movement and because of chemotactic attraction continue through the tissues to the irritating substances. If the latter are bacterial the leucocytes attempt to ingest and destroy them. Thus it can be seen that phagocytosis is an important process in the normal physiology of the body and perhaps even more so in the pathological physiology of defense against disease.

The material to be ingested by phagocytes in part determines the type of cell which participates. The macrophages are especially active in taking up bacteria, whereas the macrophages are active in ingesting inert tissue detritus. Nevertheless, macrophages often take up bacteria, as in tuberculosis, and, as has been shown by Kyes, by Bull and by Hopkins and Parker, pneumococci, typhoid bacilli and streptococci are

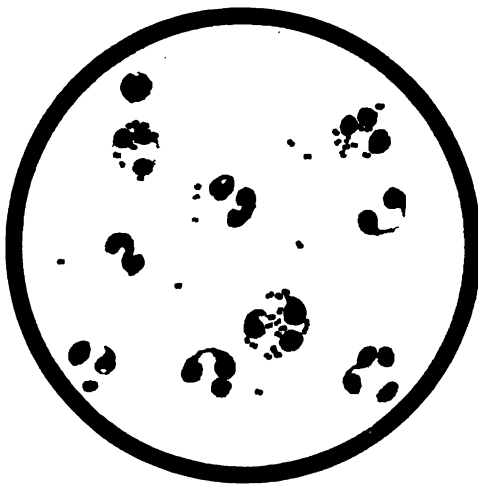


FIG. 16.—Microscopic drawing showing the phagocytosis of gonococci by the polymorphonuclear leucocytes in urethral pus.

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removed from the circulation by endothelial cells lining blood-vessels. Microphages may also play a large part in the removal of tissue detritus and may take up pigment as in malaria.

Experimental Demonstration.—The experimental demonstration of phagocytosis in mammals is comparatively simple, as the following experiment from Metchnikoff will show. The blood of a bird, such as goose, hen or pigeon, is selected because of the fact that the nucleated erythrocytes are easily distinguished from those of mammals. Defibrinated bird blood mixed with equal parts salt solution is injected (about 3.0 c.c.) into the peritoneum of a healthy guinea-pig. Material is removed for study by means of finely drawn out glass pipettes, drops being placed on slides for study with or without subsequent staining. Within the first hour the leucocytes seem to have disappeared from the peritoneum.

The disappearance is particularly striking when bacteria are injected and was interpreted by Metchnikoff as a destruction of the phagocytic cells, a phenomenon which he called *phagolysis*. At the end of from one to two hours exudate may be withdrawn which shows numerous cells, particularly macrophages. The macrophages show ingestion of the nucleated erythrocytes and at from twenty-four to forty-eight hours exhibit digestive vacuoles and partial digestion of the erythrocytes.

At the end of three days the digestion is practically complete. Metchnikoff has shown that immunization will definitely limit the appearance of phagolysis. Sanarelli, however, maintains that the disappearance of the leucocytes is not due to phagolysis but rather to the fact that the leucocytes of the peritoneal cavity and of the blood accumulate in the epiploic appendages into which the bacteria are likely to be carried by the lymphatic stream. Here, he asserts, bacteriolysis and phagocytosis progress actively. Hence the disappearance of the cells from the exudate.

A similar experiment may be performed with a suspension of pigment, as for example 5.0 c.c. finely-divided suspension of cinnabar (red mercuric oxide). This shows no digestion but active phagocytosis and a rapid transfer to regional lymph-nodes.

Phagocytosis of bacteria may be very well demonstrated with colon bacilli. It is desirable in this instance to excite some exudation before the introduction of the colon bacilli. This may be produced by injecting about twelve hours previously 10.0 c.c. sterile bouillon or aleuronat suspension. This may be done in the evening and the following morning the guinea-pig is ready for the injection of a 24-hour bouillon culture or a 24-hour slant agar culture suspended in salt solution. The subsequent phenomena are similar to those following the injection of bird blood.

The Mechanism of Phagocytosis.—In earlier experiments of this sort several questions as to the mechanism of the process arose. That the bacteria do not actively penetrate into the phagocytes has been demonstrated by direct observation of the ameboid action of the cells and is concluded also by analogy from the fact that non-motile bacteria, non-motile cells, such as erythrocytes, and inert bodies, such as cinnabar, are readily ingested by the phagocytic cells. That the bacteria are not killed before ingestion is shown by the fact that cultures may be successful in the case of anthrax bacilli shortly after they have been taken up by phagocytes. This may also be illustrated by the following experiment with the use of neutral red as a vital stain. This stains only dead cells and imparts no color to living cells. A warm hanging drop preparation of the exudate from a guinea-pig injected with colon

bacilli as outlined above may be mixed with a drop of 1 per cent. neutral red in isotonic salt solution. At first the extracellular bacteria show no stain, and but few of the intracellular bacilli take the stain. As time goes on the number of intracellular organisms taking the stain increases until they are completely digested. Metchnikoff interpreted the coloration of the bacteria as being due to an acid digesting fluid formed by the cell, but we are unable to state at the present time whether the digestion of the bacteria is due to a special ferment or due to the same ferments that digest the cells themselves after they are destroyed.

The Physical Basis of Phagocytosis.—The mechanism of phagocytosis both as regards immunity and biology in general has been the subject of much investigation. There are those who have maintained that the ameba or the leucocyte, in spite of the absence of a nervous system, exhibits individual intelligence in the selection of the material it takes up, but the bulk of experimental evidence would place the phenomenon largely on a physical chemical basis. There are important differences between free living amebæ and the phagocytes of higher animal life, such as the ectosarc and endosarc of the amebæ, its pulsating vacuoles, variety of pseudopodia, conjugation and cyst formation, but there are resemblances in movement, form, nutrition and ultimate genesis which form a basis for many comparisons. That the life activities of the ameba can be closely simulated by non-living materials has been known for many years, but the most important stimulus to these studies in recent years has been given by the work of Jennings. A fundamental conception necessary to understanding the physical basis of ameboid motion and phagocytosis is that of the phenomena of surface tension. Wells expresses the matter most clearly and concisely as follows: "Imagine a drop of fluid suspended in water—let it be a drop of protoplasm, or oil, or mercury; the drop owes its tendency to assume a spherical shape to the surface tension, which is pulling the free surface toward the center and acting with the same force on all sides. The result is that the drop is surrounded by what amounts to an elastic, well-stretched membrane, similar to the condition of a thin rubber bag distended with fluid. If at any point in the surface the tension is lessened, while elsewhere it remains the same, of necessity the wall will bulge at this point, the contents will flow into the new space so offered and the rest of the wall will contract; hence the drop moves toward the point of lowered surface tension. Conversely, if the tension is increased in one place the wall at this point will contract with greater force than elsewhere, driving the contents toward the less resistant part of the surface, and the drop will move away from the point of increased tension." The experimental demonstration of this phenomenon is relatively simple. A drop of mercury is placed in a nitric-acid solution and near it is placed a crystal of potassium dichromate. A yellow color diffuses out from the dichromate; as the color reaches the mercury the latter begins to move toward the crystal. This is the result of oxidation of the adjacent

surface of the mercury drop whereby the surface tension of this side is lowered, thus causing the progressive movement in the direction of the dichromate crystal. Similarly a drop of clove oil in a mixture of glycerol and alcohol will move about and send out pseudopodia in much the same manner as an ameba. The movement depends upon the solubility of the clove oil in alcohol, but the glycerin retards the diffusion and thus determines a certain degree of irregularity in the movements. If strong alcohol be introduced near the clove oil the surface tension of the oil is reduced and it moves toward the alcohol. Heat applied near one side of the drop will also lower the surface tension, and it moves toward the point of heat—positive thermotaxis. These experiments illustrate the physical basis of ameboid movement, but do not explain ingestion of particles. For this purpose a drop of chloroform may be placed in water and brought near a variety of objects, such as glass particles and small pieces of shellac, paraffin and glass. Such a drop will flow around a piece of shellac and dissolve it. A piece of glass covered with shellac will be taken up, the shellac dissolved and the piece of glass then extruded. If a long "hair" of shellac is brought into contact with the chloroform, the former will be bent in the middle, pseudopodia will extend along it and it will finally be curled up inside the drop and dissolved. These various activities of the oil drop or chloroform drop resemble in detail the activities of amebæ under similar circumstances and may be understood as indicating that the process of phagocytosis is based on definite physical laws. The experiments do not explain all the phenomena, however, and must be interpreted as solving the problem only partially. Various food particles are not soluble in the cytoplasm of the ameba, bacteria are not soluble in the cytoplasm of the leucocytes, but in each instance must be digested in some way. Furthermore, the phagocytes have the property of taking up inert and insoluble particles such as coal dust and other pigments, substances which cannot exert chemotaxis nor alter surface tension. The artificial ameba does not assimilate, it merely dissolves. An additional differentiation between the leucocytes and the ameba is the fact that the ameba is a free living organism capable of nourishing itself independently of life within another organism. On the other hand, the leucocyte depends upon the blood for its nutrition and differs in ameboid movement and irritability from the free living ameba. Thus we must conclude that the problem of phagocytosis is not solved by these experiments and that the life activities of these cells are not as yet explainable on a purely physical basis.

Influence of Temperature on Phagocytosis.—Madsen and his school have made accurate studies of the influence of temperature on phagocytosis. They have shown that within certain limits the phenomenon of phagocytosis increases with the degree of temperature. Starting at a point of $\pm 5^{\circ}\text{C.}$, the phagocytic power increases with temperature up to the normal temperature of the species from which the phagocytic cells are derived. In cold-blooded animals, on the other hand, the temperature of the environment within certain

limits appears to have no influence whatever on the phagocytic activity of their cells. Calderone and Runfola have recently studied the influence of temperature upon phagocytosis in the frog and find that phagocytosis proceeds actively between 5° and 40° C., but ceases when a temperature of 42° C. is reached.

OPSONINS

Introduction.—Early in the study of phagocytosis it was noted that immune animals respond to the introduction of the antigenic bacteria by a greater degree of phagocytic activity than normal animals. This was interpreted by Metchnikoff as being due to “stimulins” which were supposed to augment the activity of the phagocytic cells. The first study of importance in contraindication of Metchnikoff’s conception of stimulins was that of Denys and Leclef in 1895. They showed in a study of streptococcus immunity in rabbits that the leucocytes of normal and immune animals took up the bacteria equally well, but that both varieties of leucocytes acted much more powerfully when immune serum was added. They indicated that the process of immunization did not augment the phagocytic power of the leucocytes and concluded that in their opinion the antitoxic substance acts not upon the leucocyte but upon a poison enclosed within the bodies of the microbes or dissolved in the medium, the poison acting to protect the bacteria against the attacks of the leucocyte until neutralized by the immune substance in the serum. The observations were confirmed by other investigators and later Denys and Leclef showed that whereas extremely virulent bacteria are taken up by leucocytes in normal serum to only a slight degree, the addition of an immune serum markedly increases the phagocytosis. Little progress was made until after the discovery by Leishman whereby the study of phagocytosis could be carried out *in vitro*. Modifying this method, Wright and Douglas in 1903 published the first of a series of experiments which have built up in large measure our modern conception of the influence of serum on phagocytosis and the practical use of bacterial vaccination in the treatment of disease. They showed conclusively that it is the activity upon the bacteria of some substance in the blood which favors phagocytosis and they named the substance opsonin. By treating the bacteria with serum, then washing them to remove the serum from the surrounding medium and finally mixing with a leucocyte emulsion in salt solution they showed that phagocytosis proceeds actively. Similar treatment of the leucocytes by serum produces no augmentation of their phagocytic activity. Thus it was shown that the serum does not stimulate the leucocytes but rather prepares the bacteria so that they may more readily be ingested, hence the term opsonin (Gr. *opsono*—to prepare food). There is some variation, however, in the way the serum operates in the case of different bacteria. Tunncliffe and Davis have shown that fusiform bacilli and influenza bacilli can be taken up readily independently of the presence of serum. There are, of course, different degrees of facility with which bacteria can be taken up, varying from

those which absolutely require the intervention of an opsonin and those mentioned above, which apparently need little or no particular opsonization.

Experimental Demonstration.—For the experimental demonstration of opsonization it is necessary to have washed leucocytes, bacterial suspension and blood serum. Large quantities of leucocytes may be obtained by injecting 5.0 c.c. aleuronat suspension into a guinea-pig's peritoneum and withdrawing the exudate at the end of twelve to twenty-four hours. These may be suspended in five to ten volumes normal saline, gently mixed and centrifuged, the process being carried out three times, when the cells are said to have been washed three times. If human leucocytes are desired, 10.0 c.c. saline sodium citrate are placed in a centrifuge tube and 2.0 c.c. blood added. The tube is centrifuged at high speed, whereupon a layer or "cream" of leucocytes collects at the upper level of the cell mass. The cream can be removed by a drawn-out nipple pipette and the cells washed as indicated for the peritoneal exudate. The bacterial emulsion can be made from a twenty-four-hour slant agar culture of *staphylococcus pyogenes aureus* by adding 10.0 c.c. salt solution, allowing to stand for ten to fifteen minutes and then rotating the tube between the palms of the hands. This is pipetted into another tube and for safety may be killed by heating in a water bath at 55°–60° C. for two hours. The serum may be obtained by allowing blood to clot and then drawing off the serum. Small quantities may be obtained by the use of a tube such as shown in Fig. 8. Having these ready, 0.5 c.c. bacterial emulsion are mixed with 0.1 c.c. serum and incubated for one-half hour, then washed three times and the organisms resuspended in 0.5 c.c. saline. Several capillary pipettes are made from glass tubing (5 mm. bore) and the upper end flanged so as to take a rubber nipple. A mark is made with a grease pencil about 2 cm. from the tip, which serves as a volume indicator. (Fig. 11.) In the experiment one volume bacterial suspension, one volume bacterial emulsion and one volume serum or saline are drawn into the pipette each in succession to the mark, permitting a small amount of air to enter before the next volume is taken up so as to permit of exact measurement of the volume. These are blown into a watch crystal and mixed by blowing in and out several times; then taken into the capillary again and the end sealed. After incubation at 37° C. for fifteen minutes the tip is broken, the mixture dropped on slides or cover slips, spread, dried and stained with Wright's stain or some other modification of the Romanowsky stain. Then the number of bacteria in a given number of leucocytes (20 to 50 or more) are counted and the average calculated. A sample protocol follows:

					Average phagocytosis per cell
1. Leucocytes (washed)	+	bacteria (untreated)	+	serum	22
2. Leucocytes (washed)	+	bacteria (untreated)	+	NaCl	1
3. Leucocytes (washed)	+	bacteria (treated)	+	NaCl	14

In the above protocol it is seen that the leucocytes exhibit a slight capacity for taking up bacteria independently of the presence of serum, but that this is much augmented either in the presence of serum or by previously treating the bacteria with serum.

Normal Opsonins.—As has been indicated, the phagocytosis of bacteria and other cells is greater in immune than in normal animals, the difference being due to increase in the opsonin content of the serum of the immune animal. It was soon observed that the opsonin of the serum of normal animals could be destroyed by heat to 60° to 65° C. for 10 to 15 minutes; whereas the opsonin of immune animals is not destroyed by heat of 62° to 63° C. for forty-five minutes. Similarly exposure to light at room temperature leads to deterioration of normal opsonin in a few days but has practically no effect on immune opsonin. These differences in behavior were at first thought to constitute an

actual difference in the nature of normal and immune opsonin, but this view has now been almost entirely abandoned. In the discussion of this change of view it is essential to present first the development of work in regard to the normal opsonin. Conservative workers were not disposed to accept the opsonin as a new form of antibody and from the ease of deterioration of the normal opsonin thought that it was identical with complement. Furthermore, it was shown that fixation of complement by a hemolytic system or sensitized bacteria removes the opsonin, that yeast cells, cell detritus and bacteria will absorb both opsonin and complement, that blood serum and edema fluids contain parallel amounts of opsonin and complement, that certain body fluids, such as the aqueous humor of the eye, contain neither complement nor opsonin. Nevertheless, the removal of complement, as by heating, does not, as Hektoen has shown, remove all the normal opsonic power of the serum; and the fixation of complement by a hemolytic system or by sensitized bacteria still leaves slight opsonic power in the serum. The addition of fresh serum to a slightly active heated serum restores the activity practically to normal in much the same manner as a hemolytic amboceptor may be reactivated by complement. The following example taken from Cowie and Chapman and slightly modified serves to illustrate this reactivation. The substances indicated in the protocol are added to leucocyte and bacterial emulsions and the figures given are for the bacterial count per leucocyte:

1. Unheated (normal) serum	15.44
2. Salt solution	0.18
3. Heated serum 57° C.	1.08
4. Normal unheated serum, diluted 1 to 15	1.56
5. Heated serum + normal serum diluted 1 to 15	12.40
6. Two volumes unheated normal serum	16.08

Thus it will be seen that heating the serum reduces the phagocytic index from 15.44 to 1.08; that normal serum, diluted so that its phagocytic index is reduced to 1.56, added to heated serum, raises the index to 12.40, much higher than can be accounted for by the total indices of the two components. It can then be concluded that the normal opsonic power of serum depends upon two factors, a weakly acting thermostable element and a thermolabile element which markedly adds to the combined power of the mixture. Cowie and Chapman have shown that at 0° C. the thermostable element of opsonin is absorbed by the bacteria, but that the thermolabile element remains in the supernatant fluid and is capable of reactivating a heated serum. It has also been demonstrated by absorption experiments that the thermostable element is specific. Numerous continental workers contradict this statement, but their studies have, for the most part, ignored the existence of the thermostable element of normal opsonins. Hektoen has shown that saturation of the bacteria with opsonin and heating so as to destroy the thermolabile part leaves the bacteria in such condition that they cannot absorb any more opsonin from another serum.

Moore has found that in guinea-pigs "the complement titer varies with the opsonic index and in the same direction." These facts, together with the fact that vaccination with bacteria will increase specifically the opsonic content of the blood suggest a close similarity of opsonins to agglutinins and amboceptors. The resemblance to agglutinins is only relative for as we have seen the thermostable element of opsonin is markedly augmented in activity by the addition of fresh serum, whereas agglutinins are not affected in any way by the addition of complementary substance. Hektoen has shown that in the process of immunization the curves of opsonin and agglutinin production are nearly parallel, but that heating does not influence the agglutinin and markedly depresses the opsonic action, the latter being restored by the addition of fresh normal serum. Levaditi, in a study of the site of formation of opsonins, showed that certain organs rich in agglutinin contain no opsonins. The thermostability and specific absorption of opsonins suggest similarity to amboceptors, but the amboceptors are not capable of acting without complement whilst the opsonin is capable of acting independently of fresh serum. The fresh serum augments the activity of the thermostable element of opsonins but is not an absolute essential for activity. That the opsonin is not identical with hemolytic and bactericidal amboceptors is indicated by the fact that there are such amboceptors in sera which have no opsonic power; that in sera which show both amboceptors and opsonins there is no parallelism between the activity of the two. Sera may be strongly opsonic for certain bacteria and yet contain no bactericidal amboceptor. Much of the material quoted above has been worked out in connection with immune opsonins, but nevertheless it is safe to conclude that the opsonic action of normal serum depends upon the operation of two elements, a thermostable element which behaves as a "facultative" amboceptor and a thermolabile element which, if not identical with, resembles complement most closely.

Immune Opsonins.—As has been indicated, it is possible by immunization to increase to a very considerable degree the opsonic activity of serum. The immune opsonins were considered as of a constitution different from the normal opsonin because of the claim that the application of heat did not alter their activity. Dean showed, however, that this assumption is not true for he found that heating to 60° C. definitely though not very markedly reduces the opsonic activity of immune serum, and that reactivation takes place on the addition of a fresh normal serum. The following protocol shows the phagocytic index as determined by the use of various sera and mixtures:

Normal serum	11.9
Heated immune serum	7.1
Heated immune serum + normal serum	33.0

Hektoen reached the same conclusion with the hemopsonic power of rabbits immunized to goat erythrocytes, diluting the serum so that it

showed minimal opsonic power and no hemolytic action. One protocol from his work serves to illustrate.

Heated immune serum	Fresh guinea-pig serum	Phagocytosis
0.001	4
0.001	0.01	20
.....	0.01	0

Levaditi and Koessler showed that a serum which contained anti-complement by virtue of immunization with complement, when added to an immune opsonin, noticeably reduces the opsonic power.

The full activity of the immune opsonin depends, as can be seen, from the above experiments, upon a thermostable and a thermolabile element, as is true of the normal opsonin, but the activation by fresh serum in case of the thermostable element of immune opsonin is proportionately much less than activation of thermostable normal opsonin by fresh serum. Reference to the activation of a hemolytic amboceptor by complement shows that a given amount of complement will activate a very small amount of amboceptor in greater proportion than a large amount of amboceptor. The thermostable fraction of opsonin has been referred to as a facultative amboceptor, because the action of the thermolabile part is not essential. Assuming this interpretation to be correct and assuming that the thermolabile element operates as a complement, it is a simple matter to infer that this complement would have a proportionately larger action on the facultative amboceptor of normal opsonin, which is present in very small amount, than on the similar amboceptor of immune opsonin, which is present in relatively large amount.

Bacteriotropins.—Neufeld and his school maintain that the immune opsonin is a body which operates only in the presence of complement and that the tropins, bacteriotropins and cytotropins are bodies appearing in serum which has been rendered complement-free, and which exhibit a capacity for so altering bacteria or cells that they are easily taken up by phagocytes. Levaditi and numerous other authors agree that Neufeld has shown that the tropins are not identical with those amboceptors which lead to cytolysis, but also agree that Neufeld has not succeeded in demonstrating that the tropins are antibodies distinct and apart from the thermostable element of immune opsonin.

Opsonins for Cells other than Bacteria.—Numerous substances, including vegetable cells, such as yeasts, and bacteria, as well as a variety of animal cells, may undergo phagocytosis when influenced by opsonins. In connection with phagocytosis of animal cells the work of Hektoen and his collaborators has been most extensive. The investigations have thrown much light on the general study of opsonins and, directed particularly toward erythrocytes, have shown that the same general laws governing the phagocytosis of bacteria operate in the phagocytosis of erythrocytes. Neufeld and Händel have shown that emulsions of fat droplets in protein-containing media can serve as excitants of the formation of specific opsonic sera but conclude that in these instances the protein capsule of the fat droplets which serves to stabilize the emulsion is the important factor in the phenomenon. Led-

ingham has also shown that the injection of melanin produces a specific opsonic serum and others have shown that carbon granules, cinnabar, carmine, etc., are phagocytosed much more readily in the presence of serum than otherwise. In these latter instances it seems probable that the serum provides a protein capsule for the pigment granules, thus facilitating the action of opsonin, but at the present time no satisfactory explanation has been offered for the production of a specific immune opsonin following the injection of melanin. Neufeld and Ungermann point out the difficulty of satisfactory measurement of phagocytic action against pigment granules, and it is possible that this source of error may be sufficient to throw doubt on the results claimed to have been obtained with insoluble pigments.

Specificity and other Characters of Opsonins.—The specificity of the immune opsonins is clear-cut, as has been shown by numerous investigators. An immune opsonin produced by vaccination with staphylococci shows a marked influence on the phagocytosis of the antigenic organisms but none whatever on non-related organisms such as colon bacilli. As in the case of other immune bodies, group reactions are demonstrable. Vaccination with typhoid bacilli leads to the formation of immune opsonins which operate in high degree on the antigenic organism and also to less degree on closely-related organisms such as those of the paratyphoid groups. Dean, in working with serum dilutions in order to demonstrate that an optimum concentration of opsonin may not necessarily be found in undiluted serum, reports the following experiment. This may be interpreted as showing an inhibition zone in the stronger concentrations, although the differences are so slight as to fall within the limit of experimental error.

Dilution of serum	Phagocytic index*
0	9.7
1-2	9.6
1-4	10.0
1-8	8.2
1-16	8.5
1-32	6.4

* Average number of bacteria ingested per leucocyte.

Influence of Phagocyte and Ingested Elements.—The foregoing paragraphs have considered the influence of serum on phagocytosis, but detailed studies have shown that certain considerations in regard to both the bacteria and the leucocytes exercise some influence. Neufeld pointed out that bacterial cultures from ten to twenty-four hours old are best for *in vitro* experiments. The reaction takes place best when the bacteria are suspended in equal-parts broth and physiological salt solution, but in ordinary laboratory practice salt solution is used without the addition of broth and whatever deterring action is exercised by the salt is constant in the series of experiments. The thickness of the suspension is of importance since very thin suspensions determine a reduction in phagocytic index as compared with thicker suspensions. The optimal density of the suspensions varies with different bacteria

and must be determined, in exact work, for the organisms under investigation. The more homogeneous the emulsion, the better the phagocytosis observed. Numerous investigators have shown that under experimental conditions, bacteria killed by chemicals or by heat are phagocytosed at precisely the same rate as living organisms. Furthermore, the previous staining of the organisms has no deterrent action on phagocytosis.

Relation of Bacterial Virulence.—The relation of bacterial virulence to phagocytosis has been the subject of much research since Marchand first showed that virulent streptococci are taken up hardly at all under conditions where avirulent streptococci are phagocytosed with avidity. He demonstrated that this difference is not due to the vitality of the bacteria, for when killed by heat at 60° C., 1.8 per cent. HCl, 2.5 per cent. Na₂CO₃, or 90 per cent. alcohol, the virulent forms show the same resistance to phagocytosis. Wright and also Levaditi showed that the same difference is observable in the case of phagocytosis, without the intervention of opsonins. Rosenow confirmed Marchand's results by the use of freshly-isolated virulent pneumococci. Reduction of virulence of thirty-six strains by repeated cultivation on media resulted in increased susceptibility to phagocytosis; and a restoration of virulence by animal passages led again to decreased phagocytosis. There is, however, no absolute parallelism between virulence and susceptibility to phagocytosis. Markl, von Gruber and Futaki, as well as Lohlein and others, found that anthrax bacilli and plague bacilli when taken from culture material are actively phagocytosed *in vitro* even though highly virulent for animals. If removed from a guinea-pig's peritoneum after having grown there for several hours, they are no longer phagocytosed *in vitro*. In animal experiments they are at first the victims of active phagocytosis *in vivo*, but after several hours are resistant to phagocytosis. Proper staining shows that in the resistant stage the organisms show definite capsule formation. These experiments indicate that the resistance is entirely a function of the bacteria, but that there is some interdependence between the bacteria and the opsonin is indicated by the experiments of Ungermann, who worked with pneumococci virulent for mice in doses as small as 0.000,001 c.c., but not injurious for rabbits in doses as large as 1.0 c.c. He found that mouse serum has no opsonic action and that rabbit serum acts energetically. After repeated cultivation so as to reduce virulence for mice the organisms are opsonized by mouse serum. Von Bockstaele and also Denys and von den Bergh were able to see leucocytes in the presence of a normal serum approach and even break up chains of virulent streptococci without engulfing them; if a strong immune serum were added, there resulted active phagocytosis. In summary, these various experiments show that the possession of virulence by an organism confers upon it the power of resisting opsonization, that this power has some relation to the susceptibility of the particular animal whose serum is used for opsonization, that the resistance to opsonization is not lost on the death of the bacteria, and that in certain instances this

resistance is accompanied by capsule formation. Levaditi believes that the resistance of virulent bacteria is dependent upon some alteration of the bacterial membrane (which alteration determines in all probability the virulence of the organism) and also perhaps on the formation by the bacteria of an anti-opsonic or anti-phagocytic substance. In the latter connection Tschistowitsch and Jurewitsch claim to have shown that on washing, virulent pneumococci lose their resistance to phagocytosis, but that submitting the organisms to the action of the material in the washings restores them again to their resistant state. They considered that the salt solution removed in the washing a secretion which they called *antiphagin*. This work has not been confirmed and cannot be regarded as establishing beyond question the existence of an antiphagin.

Influences Operating upon Phagocytic Cells.—In the preliminary paragraphs of this discussion the stimulin theory of Metchnikoff was dismissed with a simple statement that such a theory exists. Nevertheless, the leucocytes and their possible alterations are of considerable importance in phagocytosis, and while it is true that increased phagocytosis resulting from immunity is not the result of stimulins, nevertheless, it is possible to augment the activity of these cells. Neisser and Guerrini gave the name *leucostimulants* to certain substances which directly act upon the leucocytes. According to Manwaring and Ruh, numerous antiseptics in proper concentration exhibit a stimulating action. According to others, calcium chloride, magnesium salts, potassium iodide, iodoform, fat soluble substances (except cholesterol), substances facilitating oxidation, pepton, quinine in certain low concentrations, nucleinic acid, similarly excite increased phagocytosis. Marbe has extracted a thermostable body from the thyroid gland which excites phagocytosis. The demonstration that the action of these various substances is upon the leucocytes depends upon the use of decreasing dilutions of the substances in the presence of sensitized bacteria and washed leucocytes.

Metchnikoff showed the influence of heat on the leucocytes in experiments which are tabulated as follows:

Degree of heat	Time of heating	Phagocytic index
40° C.	15 minutes	18
45° C.	15 minutes	8
50° C.	15 minutes	3
55° C.	5 minutes	1.2
60° C.	5 minutes	0
60° C.	30 minutes	0

In addition to heat, alterations of OH ions, alterations of osmotic pressure, cholesterol, reduction in amount of electrolytes, potassium ions, alcohols, ether, quinine and certain other of the leucostimulants in high concentrations act upon the leucocytes to depress their phagocytic activity.

Analysis of Mechanism of Phagocytosis.—The mechanism of phagocytosis includes the approach of phagocytes and the object to be

phagocytized, the ingestion of these objects and in the case of living objects their death; finally the digestion of bacteria and other suitable objects. The approach of the cells and the phagocytable objects is, according to Mesnil and his co-workers and also Levaditi, due to a physical chemical reaction and not dependent on the life of the leucocyte. If leucocytes are injured by heat to 45°, 50° or 60° C., by refrigerator temperature, by shaking, by grinding, and then mixed with bacteria and inactivated immune serum, the bacteria become clumped about the leucocytes. This reaction may be observed even if the tubes are laid in melting ice. The leucocytes that have been killed or paralyzed will not ingest the bacteria. The "anchoring" of leucocytes and bacteria will not occur unless specific opsonin is present in the serum. It occurs with fragments of leucocytes as well as other cells of the leucocyte series, such as myelocytes and myeloblasts. Thus the affinity may be expressed as existing between the protoplasm of the phagocytic cell and the sensitized bacteria or other phagocytable object.

Although actual ingestion of objects may be shown in the case of artificial amebæ it does not occur in the leucocyte unless the cell is alive and in possession of its capacity to project pseudopodia. Hence this stage of phagocytosis must be bound up with the life processes of the phagocyte.

From the earlier studies of Metchnikoff it has been known that the bacteria, after phagocytosis, are killed and digested. The influence of the blood fluids in this phenomenon has been the subject of much study and conflicting results. Metchnikoff and his co-workers were of the opinion that the leucocytes contain complement, which, as has been shown in previous chapters, is required for the action of bactericidal and bacteriolytic amboceptors. They believed that this complement is liberated only upon the destruction of the leucocytes as seen in phagolysis for they were unable to find complement in plasma. They interpreted the presence of complement in serum as due to the death of the leucocytes during clotting of the blood. This interpretation has been combated by numerous observers who have been able to demonstrate complement in plasma. In support of the conception that the death of the bacteria is due to completion of the bactericidal amboceptor-antigen complex by complement in the leucocyte, is Bordet's work with cholera vibrios. Using immune sera which contained bacteriolytic amboceptor, he found no lysis except in those bacteria that were within phagocytic cells. As opposed to this conception, the work of Neufeld and his collaborators has shown that sera may be richly opsonic without containing lytic amboceptors, and in these instances the bacteria are destroyed and digested by the phagocytes. The destruction varies with different organisms and with the virulence of the organisms, the more virulent being less readily killed than the avirulent strains. Bacteria may be cultivated on artificial media after having been ingested, a certain amount of time being necessary to kill the organisms. The act of digestion is closely bound up with that of killing the organisms. The presence of a proteolytic ferment in leucocytes has been known

since the work of Mueller and Jochmann, who placed the leucocytes of animals upon plates similar to those used for bacterial cultivation. At incubator temperature, the leucocytes exhibit distinct proteolytic power. Recent studies by Van Calcar would appear to indicate that the organs of the body which secrete digestive ferments have some influence over the ferments existing within the leucocytes. He found, for example, that after the removal of the stomach the leucocytes of the animal were unable to act as peptic digestors. Similarly the removal of the pancreas destroys the ability of the leucocytes to act as tryptic digestors. In summary it is necessary, in order to accomplish destruction and digestion, to sensitize the organisms and to have present active living leucocytes. Opsonization will not in itself kill or digest the organisms; therefore, the phagocyte must furnish some substance which either completes the action of the opsonin or of itself can kill and digest the organisms. The fact that phagocytosis in all its stages may occur in slight degree independently of opsonin would indicate that the phagocyte is the important element in death and digestion of the phagocytized object. Recent work by Bachmann would indicate that the leucocytes of normal and immune animals have a different capacity for protecting against disease. Sixty times more leucocytes from a normal animal were needed to save a guinea-pig against typhoid infection than the number required from an immune animal. In the case of anthrax the leucocytes from immune animals were eighty times more active than those from normal animals. That these studies can be interpreted as indicating a variation in the actual phagocytic power of leucocytes is open to considerable question.

It is probable that the affinity of the phagocyte and phagocytizable object is, in large part if not entirely, a physical chemical phenomenon entered into on the one hand by the cytoplasm of the leucocyte and other cells and, on the other hand, the opsonized organisms or other object. The ingestion, death and digestion are dependent upon the life function of the phagocyte, which is capable of liberating a microbicidal and microbolytic substance capable of combining with the microorganism to bring about its death and destruction.

OTHER MANIFESTATIONS OF CELLULAR RESISTANCE

Introduction.—Studies of inflammation and of other cellular activities have made it clear that body cells play an important part in resistance to disease that is not entirely explained by the phagocytic capacity of certain of the cells. As has been indicated, cells other than the polymorphonuclear leucocyte and the large mononuclear cell possess the property of phagocytosis, but this is occasional and presumably not of great importance. It seems desirable, however, to discuss the mechanisms of resistance as influenced by properties of the leucocytes other than phagocytosis, by activities of the lymphocytes and by the cells and fluids which play a part in inflammation.

Bactericidal Extracts of Leucocytes.—The destruction of bacteria within the phagocyte so impressed Metchnikoff that he assumed that

extracellular destruction is accomplished by identical destructive agents. The demonstration that extracellular destruction of bacteria (bacteriolysis) requires the participation of amboceptor and complement had little influence on Metchnikoff's views, inasmuch as he was convinced that complement originates solely in the leucocytes. As we have stated (page 129) the more recent examination of this problem makes it certain that complement exists free in the blood. Further study, more particularly of opsonins and bacteriotropins, has made it apparent that the mechanism of intracellular digestion is quite different from that of extracellular lysis. Nevertheless, the leucocytes may contribute to the extracellular destruction of bacteria. Buchner showed that the exudation, produced in the pleura of rabbits and dogs by injections of aleuronat, removed and killed by freezing and thawing, possesses the property of killing bacillus coli. Denys and Kaisin produced pleural exudates by injection of killed staphylococci and removed the cells by centrifugation. The clear supernatant fluid was actively bactericidal. Others have made extracts of exudates, and of leucocytes obtained from the blood, and have demonstrated that a bactericidal substance is to be obtained. Certainly these substances are yielded up after the destruction of the cells and, according to Petterson, they may be secreted by the cell during its life. The substances are resistant to a temperature of 56° C., but after inactivation by heat to 75° to 80° C. they cannot be reactivated by the addition of fresh extracts. This substance or group of substances has been called endolysin by Petterson and leucine by Schneider. It is not identical in all animals since that from dogs, rabbits and guinea-pigs kills bacillus proteus and bacillus anthracis, but that from the guinea-pig and cat fail to kill the bacillus typhosus and the spirillum cholerae.

Bachmann has recently reported on a so-called leucocyte antibody, "*anticorps leucocytaire*," which is distinct from the bactericidal endolysin. It appears in the leucocytes of immunized animals and may serve to produce passive immunity in other animals. It is found only in the polymorphonuclear leucocytes and may be extracted in normal serum. It is effective in protecting guinea-pigs against intraperitoneal injection of the specific organism and also acts beneficially and specifically upon established infections. A temperature of 75° C. destroys this substance, but if the material is well diluted and gelatin added, the same degree of heat serves to destroy the non-specific bactericidal substances (endolysins) but permits the specific leucocyte antibody to remain active. Bachmann believes that the persistence of this antibody in the leucocytes explains the fact that individuals retain immunity to certain diseases after the serum antibodies are no longer demonstrable.

Leucocyte Enzymes.—In contrast to the bactericidal substances extracted from leucocytes it is possible to obtain enzymes. Leber, in a study of inflammation, found that sterile pus can liquefy gelatin and the study of this proteolytic enzyme, the leucoprotease, has been extended by Müller and Jochmann, Opie, Longcope and others. This *leucoprotease* may be purified by precipitation with alcohol more par-

ticularly from glycerol extracts and the desiccated precipitate may be preserved almost indefinitely. In the moist state temperatures of from 50° to 65° C. increase its activity, but at 70° to 75° C. it is destroyed. It acts best in weakly alkaline or neutral medium, and is inhibited by acid. It differs from trypsin in that it is much less active; it does not require activation by any such substance as enterokinase, and exists within the cells in an active state rather than in the form of zymogen. It differs from the bactericidal extracts in that it cannot kill bacteria, but may digest them after their death. The blood possesses an anti-enzyme, but when the cells accumulate in bulk, as in the case of inflammatory exudates, the anti-enzyme is overbalanced and the protease dissolves necrotic cells, dead bacteria and other detritus. It is of considerable importance in the resolution of lobar pneumonia. In addition the leucocytes are stated to contain amylase, diastase, catalase, oxidase, peroxidase, nuclease and an ereptic ferment, but there appears to be a difference of opinion in regard to lipase.

Opie has described an additional ferment in areas rich in large mononuclear cells, which acts best in a very weak acid medium. It is inhibited by temperatures of 50° to 65° C., by alkali and by the concentration of HCl (0.2 per cent.) favorable for the action of pepsin. He was able to demonstrate this ferment in hyperplastic lymph-nodes rich in large mononuclear phagocytes. It is closely related to the enzymes of tissue autolysis. The acid medium which favors the action of this enzyme inhibits the activity of anti-enzyme.

Leucocyte Extracts for Therapeutic Purposes.—Pettersson noted that when leucocytes are placed in contact with blood serum for several hours the mixture is more actively bactericidal than the serum alone or salt solution extracts of the leucocytes. This led to experiments in which he injected leucocytes simultaneously with anthrax bacilli into dogs and found a moderate protection by this treatment. Opie similarly observed that the injection of leucocytes and tubercle bacilli into the pleura of dogs led to less severe manifestations than when tubercle bacilli alone are injected. Probably the most important contributions to the treatment of disease by leucocyte extracts are the studies of Hiss with the collaboration of Zinsser, Dwyer and others. Hiss obtained the leucocytes from pleural exudates produced by the injection of aleuronat suspensions. This was centrifuged before clotting occurred and the cells emulsified in distilled water. Either the leucocytes or the leucocytes and supernatant fluid were employed for treatment. From experiments with staphylococcus, pneumococcus, streptococcus, meningococcus and typhoid bacillus infections in rabbits, it was determined that protection was afforded by the extracts and that the infection was favorably influenced if therapeutic doses were given as late as twenty-four hours after infection. Encouraging results were also obtained in the treatment of human cases of pneumonia, meningitis, staphylococcus infections, erysipelas and other diseases. In analyzing the beneficial effects of this form of treatment, it was found that the bactericidal properties of the extracts are not sufficiently great to explain their

influence, they do not materially favor phagocytosis but appear to augment the migration of leucocytes to a slight degree and possibly are of importance in this way because of the fact that they exert positive chemotaxis. Zinsser states "we are inclined to believe at present that the beneficial effects of leucocyte extracts are based on the same principles as those which determine the reactions following on the injection of bacterial and any other protein." To us it appears that this method is to be included in the category of non-specific therapy previously discussed (page 30).

Specific Hyperleucocytosis.—Following upon the earlier suggestion of Bordet, Gay and his collaborators found that immune animals exhibit a much higher degree of leucocytosis following the injection of the organism to which they had been immunized than do normal animals. For example, rabbits immunized to typhoid bacilli reacted to subsequent injections of typhoid bacilli with blood counts of as high as 150,000 leucocytes per cmm., whereas normal rabbits showed a reaction of only 40,000 to 50,000 leucocytes per cmm. This phenomenon of specific hyperleucocytosis has been contradicted by McWilliams, who found no important difference in response between normal and immune animals and further states that typhoid immune rabbits react in essentially the same degree to colon bacilli as to typhoid bacilli. Others have confirmed the work of McWilliams. Zinsser and Tsen found a slight favorable difference in animals immunized to Gram negative cocci and a somewhat more marked difference in those immunized to Gram positive cocci, not in any case, however, to the degree indicated by Gay. There seems little reason for believing that a specific hyperleucocytosis plays any important part in resistance to infection. This, however, is not to be construed as an argument against vaccination, since the latter procedure is important in the production of specific opsonins, agglutinins and other immune bodies. Any response to vaccination in the form of leucocytosis must be regarded as only in small part if at all specific and is probably of the same nature as the leucocytic response to the injection of non-specific proteins and their products.

The Lymphocytes.—Lymphocytes appear in inflammatory areas as the result of infection, but accumulate in largest amounts in chronic inflammatory areas where, in most instances, the active infective agent is no longer present. The part they play in the phenomenon of inflammation and in protection against infection is not understood. From the work of Opie it seems probable that the lymphocytes may be, in part, the source of the ferment which he describes as operating in weakly acid media. As pointed out above, this ferment was obtained from hyperplastic lymph-nodes. The lymphocytes are said to contain a lipase, and it is suggested that the large collections of these cells about tuberculous foci may serve by the action of the lipase to break down the waxy shell of the bacilli. The lymphocyte is stated to possess phagocytic properties, but these are at best very slight and probably play no important part in resistance to disease. It has long been noted that the presence of tumors in the body often excites a neighboring

chronic inflammatory reaction in which lymphocytes appear in considerable numbers. J. B. Murphy and his collaborators have put to the test of experiment the hypothesis that lymphocytes are of importance in resistance to cancer. By the use of the X-ray they were able to destroy practically all the lymphoid tissue of the body of animals and found in these animals a decreased resistance to transplanted cancer. Immunity already established to cancer was also destroyed by this procedure. Similarly there was a lowered resistance to tuberculosis and to anterior poliomyelitis. In tuberculosis the lymphocyte constitutes a large element in the inflammatory reaction, and this is true also in the later stages of acute anterior poliomyelitis. Although small doses of X-ray may stimulate lymphocyte production, Murphy and his associates found that dry heat produces a more durable increase in the circulating lymphocytes. By increasing the lymphocytes in this fashion they demonstrated "the establishment of a high degree of immunity to certain transplantable cancers in mice," regardless of whether these cancers naturally showed a high or low percentage of successful inoculation. The same was found to be true in regard to the implantation of grafts from spontaneous cancers into the animals from which the grafts were removed. This subject has also been studied by F. C. Wood and associates in the Crocker Laboratory. They found that mice with lymphatic leukemia show no demonstrable immunity to tumors. They found that reduction of the total leucocyte count by means of X-ray or radium produces no increase in the successful transplantation of normal tissues. They found further that successful transplantation of the guinea-pig fibrosarcoma is not influenced by the use of X-ray. They selected an immune strain of rats, exposed them to X-ray and found no change in susceptibility to transplantable tumors. They found that the use of X-ray on rats in which a highly virulent tumor had been implanted did not prolong the life of the tumor. Wood states that "it is, therefore, evident that the lymphocyte is in no way correlated with cancer immunity." Sittenfeld also found that artificial lymphocytosis has no effect whatever on tumor growth. It is of further interest that in human cancer the lymphocytes collect about the slowly-growing rather than the rapidly-growing tumors and that the metastases are frequent in the lymph-nodes. The later experiments of Murphy on the lymphocytosis induced by heat have not received as yet extensive examination; therefore, the question remains open. Murphy's experiments are so well conducted that it is difficult to be assured that the lymphocytes play no part. The work of Wood carried out on a large number of animals is of especial significance and would indicate that the lymphocyte plays no such important part in resistance to cancer as Murphy's work appears to indicate.

Platelets.—In 1901 Levaditi noticed that following the injection of cholera vibrios they were often found clumped around small masses of platelets. The phenomenon was called thigmotropism. Govaerts subsequently demonstrated that the clumping is influenced by the action of opsonins. LeFèvre found that anti-bacterial immunization increases

thigmotropism because of the increase in activity of opsonins. Further study may throw light on the mechanism of the process, but at present its function is obscure.

The Influence of Inflammation.—Infection always produces some degree of inflammatory reaction, but this varies considerably with the type of infectious organism and with the capacity of the host to react. The exudate comprises the polymorphonuclear leucocyte, the lymphocyte, the plasma cell, the large mononuclear cell, certain other less important cells, the red blood-corpuscles, serum and fibrin. The part played by the more important of these cells is indicated above. As far as we can determine, the red blood-corpuscles appear more as an accident of the process than as an essential part of it. The fluid part of the exudate rapidly coagulates with the formation of fibrin and serum. There can be no doubt that the serum serves in certain measure to concentrate in the inflammatory areas those immune bodies qualified to offer resistance to the invader and its products. In case toxic products are present, these are diluted by the serum and the subsequent absorption of the serum with this diluted poison aids in its elimination from the body. The fibrin network probably serves in a certain measure to wall off and limit the growth of the invading organism. It also serves as a scaffolding for the support of newly-growing fixed tissue. Very early in the course of an acute inflammation the connective tissue cells proliferate. They may be phagocytic, but this property is of little significance. Certainly the most important function of the connective tissue in resistance to infection is the formation of a tissue which serves to limit the advance of the infection. The newly-growing connective tissue, with its capillaries, constitutes granulation tissue and the resistance of granulation tissue to infection is a matter of common observance. As the inflammation becomes chronic the connective tissue becomes denser and thereby provides a much less permeable wall than is found in the earlier stages of the process. The production of a local inflammation leads to the formation of an exudate which by virtue of the polymorphonuclear leucocytes opposes to infection the important process of phagocytosis; the liberation of bactericidal substances and of enzymes from the leucocytes serves to aid in resistance and to liquefy dead tissues and dead bacteria. Under favorable circumstances additional enzymes are provided by the large mononuclear cells and lymphocyte. The large mononuclears aid in the removal of dead material by virtue of their phagocytic powers. The fluid part of the exudate brings into the process the immune bodies of the circulating blood, serves to dilute toxic products and favors their absorption and elimination in dilute form. The fibrin, granulation tissue and cicatrization act as delimiting elements and operate toward the localization of the process.

CHAPTER VIII

COMPLEMENT FIXATION

INTRODUCTION.

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ACTIVATION BY COMPLEMENT.

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ANTI-COMPLEMENTARY CHEMICAL AGENCIES.

ANTI-COMPLEMENTARY ACTION OF CELLS, TISSUE EXTRACTS AND BODY FLUIDS.

ANTI-COMPLEMENTARY ACTIVITY OF IMMUNE SERA.

Introduction.—A summary of the hypotheses concerning the constitution of complements shows that there are three important views offered, namely the "pluralistic" conception of Ehrlich and Morgenroth, the "dualistic" of Metchnikoff and "unitaristic" of Bordet. As has been explained, the view of Metchnikoff that complement might be a "macrocytase" or a "microcytase" depending upon its cellular origin has been abandoned by most immunologists. Thus the conflict has been, and in certain measure still is, between the views of Ehrlich and of Bordet. Bordet and Gengou in demonstrating that the same complement is called on for bacteriolysis as for hemolysis, discovered the phenomenon named by them complement fixation ("la fixation d'alexine") which we employ in sharp contradistinction to complement deviation. The latter term implies the anchoring of complement by free amboceptor units, whereas fixation signifies the entrance of the complement into combination with antigen and amboceptor. In brief, they showed that if complement is utilized in the process of bacteriolysis it is not available for hemolysis.

The Bordet-Gengou Phenomenon.—The primary experiment was performed with plague bacilli, the serum of a horse immunized to plague bacilli, fresh guinea-pig serum (complement) and sensitized red blood-corpuscles, *i.e.*, corpuscles saturated with a specific hemolytic immune serum. They mixed an emulsion of plague bacilli, the anti-plague horse serum and complement. This mixture was left at room temperature for five hours and then the previously-sensitized erythrocytes added, the mixture incubated and observed. No hemolysis appeared, although the corpuscles were often agglutinated by the hemolytic (and hemagglutinative) immune serum. Naturally, such an ex-

periment required numerous controls, the complete series being indicated in the following protocol:

1. Plague bacilli + immune horse serum + complement + sensitized cells =
No hemolysis.
2. Plague bacilli + normal horse serum + complement + sensitized cells =
Hemolysis.
3. — — — — immune horse serum + complement + sensitized cells =
Hemolysis.
4. — — — — normal horse serum + complement + sensitized cells =
Hemolysis.
5. Plague bacilli + immune horse serum — — — — + sensitized cells =
No hemolysis.
6. Plague bacilli + normal horse serum — — — — + sensitized cells =
No hemolysis.

Throughout the experiment all the sera were inactivated except the fresh guinea-pig complement and all mixtures stood at room temperature for five hours before the addition of the sensitized erythrocytes. Hemolysis in tube 2 shows that normal horse serum does not serve as an amboceptor or sensitizer for the plague bacilli and therefore does not prevent the complement from entering into combination with the sensitized erythrocytes. Tubes 3 and 4 contain no bacterial antigen, cannot utilize complement and therefore hemolysis appears. Tubes 5 and 6 show that the bacteria are not hemolytic and that neither of the inactivated immune sera nor the inactivated normal horse serum contain complement for the completion of the amboceptor-cell complex. Bordet and Gengou showed that the same phenomenon could be observed with a wide variety of bacteria and specific immune sera both of human and lower animal origin; these operate to fix both guinea-pig and human complements, so as to prevent combination of these complements with hemolytic immune sera from several species. Furthermore, the immune sera so fixed might be specific for several varieties of erythrocytes. Muir and Martin found, however, that whereas most complements can be fixed in such experiments, this is not universally true and occasional complements are met with which do not enter into certain combinations. Furthermore, the process could be reversed so that the fixation of complement in hemolysis prevented its action to bring about bacteriolysis of sensitized bacteria. Thus it appeared that one and the same complement operates for the production of both bacteriolysis and hemolysis. This demonstration of the unity of complement has been combated by later work, and it now appears that there are certain exceptions to the rule, although it can generally be accepted.

Laboratory Demonstration of the Bordet-Gengou Phenomenon.—In order to demonstrate the phenomenon it is not necessary to use plague bacilli, as others serve the purpose equally well. The readily obtainable typhoid bacillus and typhoid immune serum can be used with good results. In setting up the test it is important to bear in mind the fact that numerous substances may interfere with the activity of complement, and among these are certain concentrations of

bacterial emulsions and extracts. Therefore, it is necessary to be sure that the amount of bacterial emulsion used in the test is not "anti-complementary," but yet in sufficient concentration to operate well. The emulsion is made from a twenty-four-hour slant agar culture (see page 81 for preparation) and may be killed by heat or formalin. The preliminary titration may be set up as follows:

Bacterial emulsion	Complement 1-10 dilution	Incubate one hour	Hemolytic amboceptor	Erythrocyte suspension	Incubate one hour	Result
0.5 c.c.	0.5 c.c.		0.5 c.c. (2 doses)	0.5 c.c.		—
0.4 c.c.	0.5 c.c.		0.5 c.c. (2 doses)	0.5 c.c.		P.H.
0.3 c.c.	0.5 c.c.		0.5 c.c. (2 doses)	0.5 c.c.		C.H.
0.2 c.c.	0.5 c.c.		0.5 c.c. (2 doses)	0.5 c.c.		C.H.
0.1 c.c.	0.5 c.c.		0.5 c.c. (2 doses)	0.5 c.c.		C.H.
0.5 c.c.		0.5 c.c. (2 doses)	0.5 c.c.		—

Each tube should be made up to a volume of 2.0 c.c. with salt solution before primary incubation. If convenient the erythrocytes may be sensitized by the previous addition of amboceptor. In the protocol C.H. indicates complete hemolysis, P.H. partial hemolysis and — no hemolysis.

The Test.—The results given indicate that 0.5 c.c. bacterial emulsion is definitely anti-complementary, but the 0.3 c.c. has no such influence. The last tube excludes hemolytic activity on the part of the emulsion. In order to be absolutely sure that the final test will not be misleading through the anti-complementary action of the bacterial emulsion it is advisable to use the next smaller amount than the titration shows to be free of anti-complementary activity, which in this case is 0.2 c.c. This being the case 2.0 c.c. bacterial emulsion may be diluted with 3.0 c.c. salt solution, whereupon 0.5 c.c. of the dilution will contain 0.2 c.c. original emulsion. The complement-fixation test may then be set up as follows:

Bacterial emulsion (2-5)	Anti-typhoid immune serum	Normal rabbit serum	Complement	Salt solution	Incubate one hour	Sensitized erythrocytes	Incubate one hour	Results
	(1-10 dilution)	(1-10 dilution)	(1-10 dilution)					
0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		—
0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C. H.
0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C. H.
.....	0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C. H.
.....	0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C. H.
.....	0.5 c.c.	1.0 c.c.		1.0 c.c.		C. H.
.....	1.5 c.c.		1.0 c.c.		—

The first incubation permits of fixation of the complement by the bacteria and their specific immune serum and the second determines whether or not complement is free to act upon the sensitized erythrocytes. For sensitization of erythrocytes the hemolytic immune serum should be diluted so that 0.5 c.c. contains two units hemolytic amboceptor, then added to an equal volume 5 per cent. erythrocyte suspension. In the above test the immune serum is diluted, so that 2.5 c.c. contain ten units amboceptor; it is then added to 2.5 c.c. 5 per cent. erythrocyte suspension and the mixture allowed to remain at room temperature for one hour. The protocol given above shows, reading from below upward, that the hemolytic immune serum used for sensitization is not of itself hemolytic, that the complement is in sufficient concentration for hemolysis, that neither the bacterial emulsion nor the typhoid immune serum is anti-complementary in the amounts used. In the first tube the bacterial emulsion, specific anti-bacterial serum and complement interact so that the complement is not free to combine with the sensitized erythrocytes, whereas tube 2 shows that normal rabbit serum will not fix complement.

Specific Character of the Test.—In order to elaborate the test and to show its specificity it is well also to titrate an emulsion of some other organism, for example, colon bacilli for anti-complementary activity at the same time the typhoid emulsion is titrated and in the same manner. If this shows anti-comple-

mentary activity in a dose of 0.3 c.c., then 0.1 c.c. is used in the test. The fully controlled test would then be set up as follows:

Typhoid emulsion (2/5)	Coli emulsion (1/5)	Anti-typhoid immune serum (1/10 dilution)	Complement (1/10 dilution)	Salt solution	Incubate one hour	Sensitized erythrocytes	Incubate one hour	Results
0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		—
0.25 c.c.	0.5 c.c.	0.5 c.c.	0.25 c.c.	Incubate one hour	1.0 c.c.	Incubate one hour	—
.....	0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C.H.
0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C.H.
.....	0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C.H.
.....	0.5 c.c.	0.5 c.c.	0.5 c.c.		1.0 c.c.		C.H.
.....	0.5 c.c.	1.0 c.c.		1.0 c.c.		C.H.
.....	1.5 c.c.		1.0 c.c.		—

Reading from above downward, the second tube shows 0.25 c.c. typhoid emulsion diluted 2 to 5, thus corresponding in bulk of original emulsion, to the bulk of coli emulsion in 0.5 c.c. of a 1-5 dilution. It is necessary to use the smaller bulk of coli emulsion in order to prevent anti-complementary activity. Both quantities of typhoid emulsion are sufficient to fix the complement, whereas the coli emulsion (tube 3) does not. The next three tubes which are controls show that neither typhoid emulsion, coli emulsion nor anti-typhoid immune serum have any anti-complementary activity. The last two tubes show that the complement is in sufficient concentration to operate and that the sensitized erythrocytes will not of themselves hemolyze under the conditions of the experiment. If the results prove to be confusing it is necessary to make additional controls to determine if any of the reagents is hemolytic. This contingency is extremely rare if proper care is given in their preparation. The test in this form shows that the reaction is specific.

Inhibition Zones.—The phenomenon of complement fixation exhibits certain of the characters noted in regard to other immune reactions, not only in the titration of the reacting bodies but also in the formation of the so-called inhibition zones and in the group reaction. These latter features are best illustrated with the fixation of complement by immune sera prepared from the use of dissolved protein. Gengou, a year after the publication of Bordet and Gengou, showed that the inoculation into an animal of dissolved proteins, such as egg-white, could lead to the formation of bodies which participate in complement fixation with the specific antigen. This was confirmed by Moreschi and later by Neisser and Sachs. The latter authors applied the reaction to the forensic determination of protein. Gengou was of the opinion that the immunization of animals with dissolved protein led to the formation not only of precipitins but also of complement-fixing bodies. The relation between these two immune substances will be discussed after presenting data concerning inhibition zones and group reaction. An experiment from the work of Neisser and Sachs serves to illustrate the fact that immune serum may be used in the reaction in such strong concentration as to inhibit fixation of the complement. For this purpose they arranged two series of tubes. In series A they placed decreasing amounts of the specific immune serum, a constant quantity of 0.2 c.c. of 1-2000 solution of the antigenic human serum and 0.1 c.c. fresh guinea-pig complement. In series B the same constituents were placed with the exception of the immune serum which was replaced in each tube by 0.2 c.c. salt solution. These mixtures were incubated at 37° C. and then sensitized red blood-corpuscles were

added, followed by another period of incubation. In this particular instance they employed ox blood-cells and immune serum prepared by injection of ox blood-cells into the rabbit.

MODIFIED PROTOCOL FROM NEISSER AND SACHS		
Immune serum 1-10 dilution	Series A	Series B
1.0 c.c.	+	—
0.75 c.c.	+	—
0.5 c.c.	+++	—
0.35 c.c.	+++	—
0.25 c.c.	+++	—
0.15 c.c.	++	—
0.1 c.c.	+	—
0.0 c.c.	—	—

The above protocol shows that in the strong concentration of immune serum the fixation of complement is not as marked as in somewhat weaker concentration. Nevertheless, there also comes a point when the concentration is too dilute to permit of fixation. The tubes in series B show that the concentration of human serum in itself is not sufficiently great to prevent the hemolytic reaction. Two points are of interest in this connection. In the first place, it is possible to add immune serum or other serum in amounts so large that the serum itself will have inhibitory action upon the complement. Under optimal conditions immune serum may be diluted to an extreme degree and still act as a complement-fixing body; for example, Friedberger, by the use of a well-prepared serum was able to demonstrate complement fixation by an immune serum diluted 1-1,000,000,000. The same delicacy has not been confirmed by other investigators and must be regarded as a scientific curiosity. The dilution of the antigenic protein can be carried to a considerable degree but not usually to the same degree as is possible with antiserum.

Group Reactions.—In the application of the complement-fixation test to the forensic determination of dissolved protein, Neisser and Sachs showed that the group phenomenon also appears. They also showed that the antigenic serum could be very much reduced in amount and still give complement fixation. The following protocol illustrates the manner in which such a demonstration may be made. In setting up the test there was used throughout a constant quantity of 0.1 c.c. immune serum prepared by the injection of human serum. The antigenic serum was added according to the amounts indicated in the protocol. Complement was used in amounts of 0.05 c.c. The mixtures were incubated and then beef blood-corpuscles which had been sensitized with a specific anti-beef corpuscle serum were added, the mixtures again incubated and the degree of fixation determined.

GROUP REACTION MODIFIED FROM NEISSER AND SACHS			
Amounts of antigenic serum	Man	Fixation with serum of Monkey	Goat
0.01	+++	+++	—
0.001	+++	+++	—
0.0001	+++	+	—
0.00001	+	—	—
0.000001	—	—	—
0.0	—	—	—

The above protocol shows that anti-human serum is capable of fixing complement in the presence of an amount of antigenic serum, which is considerably less in the case of human antigenic serum than in the case of monkey antigenic serum. Thus the group reaction is indicated by the fact that the serum of a closely-related species is in certain doses sufficient to produce fixation. Muir and Martin, also, were able to demonstrate similar reactions in several different animal groups.

Relation of Complement-fixing Bodies to Other Immune Bodies.—The fact that the treatment of animals with a protein in solution can lead to the development in the animal's serum of a capacity both for precipitating the antigen and combining with the antigen to fix complement suggests naturally that there may be some relationship between the two phenomena. Gay and Moreschi independently were able to show that precipitates formed by the action of a specific immune serum can so bind complement as to prevent its action upon a hemolytic system. The assumption is justified, therefore, that the two phenomena are very closely related and may indicate that complement fixation depends in part at least upon fixation of the complement by a precipitate. The question naturally arises then whether or not there may be complement fixation without precipitation or precipitation without complement fixation. Furthermore, a fundamental problem is whether or not the two activities of the antiserum depend upon two different immune bodies in the serum or upon the double capacity of the same immune body. Neisser and Sachs were able to show that complement fixation occurred with very much smaller amounts of antigen than does visible precipitation. As has been mentioned before, in reference to the delicacy of the reaction, it was pointed out that fixation of complement may occur with dilutions of 1–1,000,000,000, whereas visible precipitation has never occurred in such marked dilution of antigenic or of immune serum. Thus it can be concluded that the presence of a visible precipitate is not necessary for the fixation of complement, a statement amply corroborated by Muir and Martin. Wassermann and Bruck found that by permitting bacterial extracts to stand for a considerable time, the extracts were no longer precipitable in the presence of specific precipitating immune sera, whereas fresh extracts show beautiful precipitation. Nevertheless, both new and old bacterial extracts were found to fix complement in the presence of the specific immune serum. Liefmann further showed that the action of heat may so alter the antigenic protein as to lead to differences in complement fixation and precipitation. He immunized rabbits with egg-white and found that after heating the egg-white it could be so changed that it was no longer precipitable by the immune serum but could still operate with the immune serum in complement fixation.

Felke and also Garbat have found that anti-typhoid vaccination in man leads to the production of agglutinins, but to no or very slight production of complement-fixing bodies. Felke found that in the course of typhoid fever and during convalescence complement fixation could

be demonstrated in addition to agglutination. Most of the preceding experiments indicate that the phenomena of precipitation and complement fixation are not necessarily associated, but, on the other hand, cannot be interpreted to indicate that the immune serum contains two different immune bodies. Friedberger and Liefmann, working independently, showed, however, that heating an immune serum to 67° C. can destroy the precipitin in the serum without altering the capacity of the serum for participating in complement fixation. This experiment has been interpreted as indicating that precipitating and complement-fixing bodies represent independent activities but not necessarily that they are different bodies. Muir and Martin found that upon immunizing animals they were able to demonstrate that the serum of these animals contained complement-fixing powers earlier than precipitins could be demonstrated. Altmann found that complement-fixation bodies appeared earlier than agglutinins for paratyphosus B and colon bacilli but with the use of typhoid bacilli both bodies appeared about the same time. As a converse of this demonstration, Moreschi immunized birds with rabbit serum and found in contravention to his earlier work that he was able to produce a precipitin of very high titer without being able to demonstrate the power of complement fixation on the part of the immune serum. This was corroborated by Sobernheim. Liefmann was able to show a certain amount of difference in the activity of immune serum. He brought the immune serum in contact with the antigen at 0° C. for sufficient time to produce a considerable amount of precipitate. He then centrifuged the precipitate and found that the supernatant fluid at 37° C. was capable of fixing complement. Lebailly, by the fractional addition of antigen to the precipitating immune serum, was able apparently to separate the precipitating and complement-fixing bodies. Arlo precipitated the antigenic and immune sera by means of CO₂, thereby obtaining the globulins in the precipitate. In both instances the complement-fixing body was found in the redissolved globulin fraction and the precipitating body was found in the supernatant fluid. This has been controverted by Bruynoghe, who maintains that euglobulins are capable of producing non-specific fixation. Reviewing all this experimental evidence, it seems perfectly clear that complement fixation can and does occur independently of visible precipitation, a statement supported by a great mass of more recent investigation of the subject. None of these experiments, however, can be safely interpreted as indicating that there are two separate bodies in the immune serum. Neufeld and Händel, however, appear to be definitely of the opinion that there are two separate bodies concerned. They found that sensitized cholera spirilla are capable of fixing the hemolytic complement at 0° C. but that at 37° C. the organisms will fix both hemolytic and bacteriolytic complement. They explain this by assuming that the fixation at higher temperature is due to the bactericidal amboceptor but that the fixation at 0° C. is due to a separate substance which they named the Bordet antibody. Such experimental evidence cannot be accepted as final. Sachs states that *a priori* it can

be supposed that the antigenic protein can simultaneously combine with precipitins and with amboceptor. He offers the hypothesis that one immune molecule may contain different binding complexes, one, for example, combining with precipitins to produce a precipitate and the other combining with the antigen and the complement to produce fixation. If this view be accepted, the experiment of Friedberger and Liefmann, in which the immune serum was heated, indicates that of the two binding complexes the precipitating one is the more labile. It can very readily be seen that the interpretation of Sachs depends almost entirely upon an acceptance of the Ehrlich hypothesis of the structure of immune bodies.

Dean has examined the question and finds that the optimal relationship between antigen and immune serum for the production of precipitation is by no means necessarily the optimal relationship for complement fixation. Therefore, the two phenomena, as has already been pointed out, are by no means parallel. He is of the opinion, however, that this lack of parallelism is not necessarily an indication that the two things are entirely distinct and separate. He is of the opinion "that they represent two phases of the same reaction." The complement fixation represents the earliest and more delicate stage of a reaction which, in its more marked manifestation, is seen by the formation of a precipitate. Zinsser has studied the matter carefully and has come to the conclusion "that the precipitation is merely a secondary, colloidal phenomenon, which may, or may not, coincide with the phase of greatest alexin (complement) fixation, according to other fortuitous conditions which may favor or retard flocculation." He found that a mixture of sheep serum and its specific immune serum showed complement-fixing activity only in the precipitate. On the other hand, in a mixture of a filtrate of typhoid bacilli and a specific immune serum both the precipitate and the supernatant fluid were capable of fixing complement. "From this it seems to follow that immunization with the more complex cellular elements has given rise to the precipitating antibodies present also in the anti-sheep serum, and in addition to this to sensitizers which are not precipitable (remaining in the supernatant liquid) and not present in the anti-sheep serum." He, therefore, is of the opinion that since both the antigen and the immune body are colloidal in character they may be expected to follow the laws of colloids. This may be interpreted to indicate that the contact of the mutually precipitating colloids must be present in optimal concentration in order to show a visible precipitation, but, on the other hand, the interaction of the two bodies which, in the quantities employed, show no visible precipitate, may be demonstrated by the complement-fixation test. He states "that the visible precipitation would seem, therefore, to be a secondary phenomenon, the essential one being the union of an antigen with a sensitizer by which it is rendered amenable to the action of the alexin" (complement).

Is the Complement-fixing Body an Amboceptor?—There arises further the question as to whether or not the body, which, in combina-

tion with antigen, serves to fix complement, is to be regarded as an amboceptor (sensitizer). As has been shown in the discussion of cytolysins, it is possible at 0° C. to bring about a selective combination of hemolytic amboceptor with its antigen. Liefmann attempted to bring about a union of complement-fixing body and its antigen in this way but was unsuccessful. It is known that if a considerable excess of antigen or antiserum is present, complement may also be absorbed at 0° C. and in such an experiment as Liefmann's it is impossible to say that such an excess did not exist. Therefore, the experiment is not conclusive. Neufeld and Händel also attempted selective absorption at 37° C. They showed that cholera vibrios and their specific immune sera fix the hemolytic complement at 0° C., whereas the bacteriolytic complement remains active. At 37° C. both complements are fixed. They are of the opinion that at 0° C. the complement-fixing amboceptor is bound to the hemolytic complement and that at 37° C. both the complement-fixing and bacteriolytic amboceptors are active. This experiment has been held to support the hypothesis of the multiplicity of complements. They also found that an immune serum produced by the injection of a certain water vibrio acted as a complement-fixing body with cholera spirilla but did not serve as a bacteriolytic amboceptor. This may be interpreted as indicating that the two immune bodies are distinct but does not prove the amboceptor nature of that body which enters into the phenomenon of complement fixation. It may very well be that the experimental conditions were not optimal to the reactions and that while investigators sought to separate two forms of complement they were working with one and the same body which operates somewhat differently under the diverse conditions. Sachs interprets the amboceptor as a body which brings about the union between antigen and complement but states that certain amboceptors may be toxic (lytic) and others, for example, those serving to fix complement, may be considered as atoxic. He considers that the differences in effect may be the result of a number of factors, including mass action and differences in combining avidity of the various reacting bodies. It would appear to us that Zinsser's interpretation in regard to precipitins might also be applied here and that the lysis of cells may be an incident in complement fixation, certain conditions favoring lysis, others merely fixation of complement. If this be accepted, the complement-fixing body must be regarded as an amboceptor or sensitizer in the same sense as are the cytolysins.

Activation by Complement.—The utilization of complement in hemolysis serves so to fix complement that it cannot activate a bacteriolytic amboceptor. Therefore, hemolysis exhibits the fixation of complement in association with lysis of the cells. Händel found that hemolytic and complement-fixing properties of an immune serum were parallel, but Muir and Martin observed marked differences. The latter investigators produced two immune sera, one against ox serum and the other against ox cells, both of which exhibited hemolytic and complement-fixing properties. The immune serum prepared against ox cells

laked the antigenic cells in doses of 0.0015 c.c. and fixed complement in the presence of 0.001 c.c. ox serum. The immune serum prepared against ox serum hemolyzed ox cells in doses of 0.05 c.c. but fixed complement when combined with only 0.000,001 c.c. of ox serum. The immune serum against ox serum had only about one-thirtieth the hemolytic power of the immune serum prepared against ox cells but was 1000 times more powerful in fixing complement. They found that ox cells can absorb hemolysin from an immune serum without removing the precipitating or complement-fixing activity and conclude, in opposition to the hypothesis offered at the end of the preceding paragraph, that the complement-fixing body and the hemolysin are distinct and separate immune bodies.

Fixation of the Complement of Natural Hemolysins.—In the case of natural hemolysins the complement in many instances is apparently in a state of close combination with the thermostable lytic body. The entrance of such complements into the phenomenon of complement fixation has only rarely been demonstrated and then only in the case of those naturally hemolytic sera in which it is possible to absorb hemolytic amboceptor at 0° C. without at the same time removing the complement.

Nature of Antigen and Amboceptor.—The chemical character of the antigen and amboceptor have been studied more particularly in connection with investigations of the Wassermann test and will be considered in the discussion of that application of complement fixation. It may be said at this place, however, that the complement-fixing immune body will resist the ordinary inactivating temperature of 56° C. and is therefore to be regarded as thermostable but is destroyed by 75° C. for one hour. The antigen is thermostable in the same sense but is reduced in activity at 75° C. but not destroyed until 100° C. is reached.

Inhibition of Complement other than by Fixation.—Of great importance are the factors that exercise an influence upon complementary activity. Those which operate on the living animal have been discussed in the chapter on Cytolysins (see page 127). There was also presented a brief discussion of physical conditions such as heat, exposure to light, desiccation, etc. All these factors must be considered in interpretations of complement fixation, and in addition it is considered desirable to present certain other conditions which may be gathered into three classes (*a*) chemicals, (*b*) various tissues and fluids, (*c*) antisera.

Anti-complementary Chemical Agencies.—The salt concentration of the media for complement fixation is extremely important and reaches its optimum at a point isotonic with the body fluids. The action of complement is decreased in hypotonic and absent in salt free media. Examination of this phenomenon leads to the conclusion that such action is upon complement rather than upon amboceptor, and Ferrata is of the opinion that the important change is the splitting of the complement into mid-piece and end-piece. Under these circumstances the mid-piece may be bound to the amboceptor-antigen complex, but as

the end-piece remains free, complementary activity does not appear. This explanation, however, is only hypothetical, is not entirely supported by other experiments and fails to take into account the influence of salts on colloidal suspensions and solutions. Excesses of salts also interfere with the action of complement, but on dilution to isotonicity the function is immediately restored. Therefore, the salts do no permanent injury to complement. Hektoen and Reudiger, as well as Manwaring, offer the explanation that ionization of the salt permits of a union with complement which is easily reversible. Certain salts, such as those of bile acids, as well as sodium oleate, permanently injure complement. The salts are of themselves hemolytic, but serum inhibits their hemolytic activity. The amounts which are hemolytic in themselves completely inhibit complement and by virtue of the presence of serum cannot produce lysis.

Acids and alkalis in considerable concentration permanently destroy complement, but if the injury be due to a dilute alkali the complementary activity may be restored by neutralization. It appears that moderate concentrations of acids destroy complement without restoration by neutralization. Dilute acids accelerate hemolysis and for this reason are to be avoided in accurate work with complement fixation. Certain protein products, such as urea (also urea sulphate) and guanidin are anti-complementary.

Colloids may also inhibit complement as, for example, the organic colloids, glycogen, inulin, pepton, albumose, gelatin, etc., as well as inorganic colloids, such as quartz sand, kaolin and carbon. Numerous indifferent chemical precipitates, such as colloidal iron hydroxide and protein precipitates inhibit complementary activity. It is possible that in certain measure this may depend upon their interference with the complement amboceptor and antigen behaving as interacting colloids.

The influence of lipids on complementary activity is of great importance, particularly in the Wassermann test, but we may mention at this point that lecithin, cholesterol, protagon and tristearin in sufficient concentration are anti-complementary as well as certain lipins, including the neutral fats, olive oil, triolein, etc. Added, finally, to the list of chemical agents are boric acid, benzoic acid, formalin, sodium fluoride, sodium sulphite and extracts of certain spices.

Anti-complementary Action of Cells, Tissue Extracts and Body Fluids.—As was pointed out by von Dungern, most animal cells either in the form of emulsions or cells may inhibit the activity of complement. Muir found that the stroma of red blood-corpuscles enters into fixed combination with complement and that if washed red corpuscles are heated to 55° C. for twenty-four hours they also will combine directly. The union does not take place at 0° C. but occurs readily at 37° C. The combination is apparently not dissociable. Not only animal cells but also a wide variety of bacterial emulsions or their filtrates as well as yeast cells fix complement. On the basis of the Ehrlich hypothesis this may be due to the union of complement with the complementophile groups of those sessile receptors of cells which by im-

munization are overproduced and become free in the blood. Other investigations, particularly those of Landsteiner and von Eisler, indicate that the cell lipoids play a part in the union with complement. The material extracted from the cells by petroleum ether was found to be definitely anti-hemolytic and furthermore this was especially true if the cells used in hemolysis were from the same species as the lipoidal extracts. Landsteiner and von Eisler demonstrated in addition that cells treated with fat-dissolving agents were less susceptible to hemolysis than normal cells. They suggested the possibility that the fixing substance may be a lipid protein combination. Bang and Forssman extracted cells with ether and found that an acetone soluble material could be recovered that was definitely anti-complementary. Dantivitz and Landsteiner confirmed this but found in addition that the fraction remaining in the ether, the acetone insoluble fraction, could fix normal amboceptor but not immune amboceptors. Thus it will be seen that the finer details of the anti-hemolytic powers of lipoidal extracts are still unsettled. As to the anti-complementary action of bacterial extracts Zinsser suggests that it may be non-specific and comparable to the anti-complementary activity, mentioned in the previous paragraph, of such inert substances as kaolin and quartz sand.

The body fluids of importance in this connection are the tissue juices, certain pathological exudates and more particularly the blood serum. Camus and Gley found that a normal hemolysin may be inhibited by the addition of a similar serum which had been inactivated. Müller showed that a heated serum may inhibit the activity of other sera, and concluded that this was due to an anti-complementary activity. Extreme instances of this action have been reported by Kenneway and Wright. Muir and Browning demonstrated that inactivated sera homologous with those used as complement were more strongly anti-complementary than heterologous sera. They concluded that the action was due to the presence in the heated sera of complementoid which, at least partly, excluded the complement from union with the amboceptor. Bordet and Gay found that a sufficient dilution of inactivated sera removed the anti-complementary action and therefore consider concentration of the serum a most important factor. This would indicate that the inhibition is, in general, against the reaction, although Sachs offers the suggestion that the dilution provides for a dissociation of complement and anti-complement. More proof than is now at hand is necessary in order to admit the existence of an anti-complement in the sense in which Sachs uses the term. Of great importance is the work of Noguchi, who found that whereas heating the serum to 56° C. permits of the demonstration of anti-lytic powers, a temperature of 70° C. considerably augments this activity. Noguchi was able to extract from both serum and cells by means of ether a substance, highly thermostable (90° C.), which exhibited the same anti-lytic properties as the serum. The removal of the ether extract left the serum free from anti-lytic activity. He named the substance "protectin" and believed it to be the source of the inhibiting action of serum. Noguchi's

opinion is that the inhibiting action of serum is largely anti-complementary in nature, although in part the action may be upon the amboceptor. The great thermoresistance of the body in the serum argues against the assumption of an anti-complement in the strict immunological sense. The action may well be anti-complementary, but from the work of Bordet and Gay, as well as of Noguchi, it would appear that the concentration of colloids associated with a disturbance of lipoidal balance or combination must occupy a most important place in hypotheses concerning this phenomenon. Of practical importance is the fact that prolonged preservation of serum increases its anti-lytic capacity.

Anti-hemolytic Activity of Immune Sera.—In discussing the properties of complement (see page 137) we mentioned the experimental evidence concerning the production of anti-lysins and anti-complements by the injection of immune and normal sera. The anti-lytic activity of such immune sera was thought at first to be due to an anti-complement, but later was thought to be the result of action upon the amboceptor or sensitizer. It must be recognized, however, that the injection of a serum, whether it contain complement or immune amboceptor, leads to the production of a precipitin and that such precipitins can be demonstrated in the immune sera containing the so-called anti-complement. As has been pointed out in the preceding discussion on complement fixation the presence of precipitates serves to fix complement and this probably accounts for the anti-lytic and anti-complementary powers of the immune sera.

The fact that agglutination of the red cells inhibits their lysis was pointed out independently by Händel and by Karsner and Pearce. This renders inadvisable the use for complement-fixation tests of sera which are strongly hemagglutinative.

See also p. 140.

CHAPTER IX

APPLICATION OF COMPLEMENT FIXATION TO THE DIAGNOSIS OF DISEASE

THE WASSERMANN REACTION.
INTRODUCTION.
THE ANTIGEN.
NATURE OF THE SYPHILITIC ANTIGEN.
THE SYPHILITIC "AMBOCEPTOR."
NATURE OF THE SYPHILITIC "AMBOCEPTOR."
THE COMPLEMENT.
THE HEMOLYTIC SYSTEM.
PRESERVATION OF ERYTHROCYTES.
INFLUENCE OF TEMPERATURE UPON THE REACTION.
TECHNIC OF THE TEST.
THE ANTIGEN.
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INTERPRETATION OF RESULTS.
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TEST OF SPINAL FLUID.
POST-MORTEM WASSERMANN TESTS.
COMPLEMENT FIXATION IN TUBERCULOSIS.
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COMPLEMENT FIXATION IN GONOCOCCUS INFECTIONS.
OTHER COMPLEMENT-FIXATION TESTS.
GLANDERS.
TYPHOID FEVER.
SMALLPOX.
WHOOPIING COUGH.
ECHINOCOCCUS CYST.
MALIGNANT TUMORS.
SPOROTRICHOSIS.

THE WASSERMANN REACTION

Introduction.—The demonstration of complement fixation employs five reagents, syphilitic antigen, red blood-cells, syphilitic serum, hemolytic serum and the complement. Having any four of these known it is possible to determine the immunological nature of an unknown fifth reagent. This unknown may be an antigenic substance or may be an amboceptor. In the forensic tests for species proteins the unknown is the questionable protein which is employed as an antigen; in other tests the unknown may be bacteria or bacterial proteins. In the Wassermann and other clinical tests the unknown is an amboceptor or similar substance, produced in the blood and other body fluids of the diseased subject.

After preliminary experiments on animals, Wassermann, Neisser, Bruck and Schucht published in 1906 the results of a series of complement-fixation tests in cases of human syphilis and demonstrated the

clinical value of the reaction. The widespread use of the reaction has led to marked advances in the understanding of this disease, its sequelæ and its treatment. This application of the Bordet-Gengou phenomenon has enabled science to progress far toward the elimination of one of the greatest plagues of mankind. Wassermann and his collaborators had first shown that the Bordet-Gengou phenomenon was applicable not only to bacterial suspensions but also to bacterial extracts and from this developed the proposition that the causative agent of syphilis might act as an antigen in extracts from syphilitic organs. The test was originally performed with a salt solution extract of the liver or spleen of a syphilitic fetus (rich in *treponema pallidum*), inactivated human serum, guinea-pig complement, an inactivated hemolytic immune serum and sheep erythrocytes. All the reagents were tested and titrated to avoid factors of error and proper controls were instituted in each experiment. Much has been accomplished by further study in the hands of numberless investigators, but we shall limit our discussion to those features which are of fundamental importance in the understanding and application of the test.

The Antigen.—The preparation of the antigen is one of the most important features of this test. It would be supposed that an extract of a pure culture of the *treponema pallidum* should give the most specific results. This, however, has not proved to be the case. It is difficult to grow the organism in pure culture and the method of cultivation interposes difficulties in the way of obtaining pure extracts. Results are variable and therefore not so specific as with the use of other antigens. Until recently the organism had not been cultured *in vitro* and Wassermann and many of his successors were unable to utilize the method. Wassermann selected the organs of syphilitic fetuses, because they were known to contain large numbers of *treponemata*, and from these made extracts in physiologic salt solution. He cut syphilitic fetal liver in fine pieces and mixed 100 grams liver with 360 c.c. physiologic salt solution and 40 c.c. 5 per cent. phenol solution. This was shaken for twenty-four hours, centrifuged and the supernatant fluid employed as antigen. Practical experience shows that these antigens vary considerably in strength and rapidly lose fixing power. Deterioration may result from light, air, warmth and freezing, so that the extract must be kept tightly stoppered in the dark at low but not freezing temperature. Marie and Levaditi dried and pulverized the liver in order to preserve it and made up salt solution extracts when needed. Morgenroth and Stertz preserved the organ in the frozen state. The subsequent work of Weil and of Lansteiner and their colleagues indicated that tumor extracts, extracts of animal tissues and of normal human tissues would operate as antigens. More recently Varney and Baeslack have employed extracts of experimentally inoculated testes of the rabbit in that stage of infection when the organs are richly infiltrated with the *treponema*.

Landsteiner, Müller and Pötzl found that alcoholic extracts of guinea-pig heart serve admirably as antigen. Independently Porges

and Meier showed that alcoholic extracts of normal or syphilitic fetal organs operate equally as well as the watery extracts of syphilitic organs. These studies demonstrated that the antigen in the Wassermann test is not necessarily derived from the *treponema pallidum*, is alcohol soluble and therefore is largely of lipoidal nature. Landsteiner, Müller and Pötzl extracted 1. gram heart with 50. c.c. absolute alcohol but this method has been somewhat modified. For practical purposes 50 c.c. absolute alcohol are placed in a wide-mouth amber bottle and as guinea-pigs are killed in the laboratory the heart is freed from blood and connective tissue, cut into a few pieces and placed in the alcohol. When ten hearts are so collected they are dried and ground in a mortar. Ten grams of the dried powder are returned to the alcohol and the volume made up to 100. c.c. This is shaken for twelve hours and placed either at 60° C. for about twelve hours or at 37° C. for about five days. It is then filtered and the filtrate preserved in a cool, dark place. Further, a second extraction with alcohol of the first dried extract yields an antigen of greater value because it contains less lytic and anti-lytic substance, although it may be slightly weaker in fixing power. Apparently, however, the alcoholic extracts of syphilitic organs produce more specific antigens. To prepare such an antigen 100 grams syphilitic liver are freed from surrounding tissue, washed free of blood and cut into fine pieces. This is extracted in 1000 c.c. absolute alcohol for a week at 37° C., the flask being shaken several times daily. It is then filtered and titrated.

Porges and Meier found that lecithin could, within certain limits, be substituted for the antigenic extracts. This naturally led to extensive investigation of the nature of the substance or substances concerned. The fact that ether extracts of alcohol soluble antigen, according to Levaditi and Yamanouchi, did not contain antigen led to the thought that salts of bile acids might serve as antigens. Neither lecithin nor salts of bile acids give consistent results in the actual test and at the present time no pure substance serves well as antigen. The importance of lecithin was further emphasized by the refined technic of Noguchi in preparing the so-called acetone insoluble antigen. This method appears to be especially adapted to the use of normal human organs, particularly heart. The tissue is cut into fine pieces, mixed with five times its weight of absolute alcohol and placed at 37° C. for from five to seven days. It is then filtered and the clear filtrate evaporated in a dish by means of an electric fan or in a vacuum desiccator. The residue is taken up in as small a volume of ether as will permit solution and allowed to stand overnight. The clear supernatant fluid is decanted and slightly evaporated. To it is added four volumes of acetone. The supernatant fluid is poured off and the precipitate allowed to evaporate to a resinous consistence. Three-tenths of a gram of this mass is added to a mixture of 1.0 c.c. ether and 9.0 c.c. pure absolute methyl alcohol and preserved in a dark, cool place. According to certain reports, it would appear that this antigen gives positive results in cases which are negative with other antigens and in which syphilis has not

been demonstrated by clinical examination. It is useful, however, as a control of other antigens with which doubtful results have been obtained.

The source of the lecithin appears to play some rôle in its value as an antigen; that from heart is most active, while that from liver, brain and egg yolk follow in the order named. An extract such as that recommended by Noguchi contains in all probability a mixture of lipoids and unsaturated fatty acids; Noguchi and Bronfenbrenner found the fixing capacity of such extracts to vary in accordance with the content of unsaturated fatty acids. Browning and Cruikshank found that the addition of cholesterol to the antigen augments the delicacy of the reaction and this method has found widespread use in this country, particularly through the work of Walker and Swift. The latter investigators recommend the addition to alcoholic extracts of human or guinea-pig hearts of 0.4 per cent. of cholesterol. In the hands of several workers this has so increased the fixing power of the antigen as to give positive results in the presence of non-syphilitic serum, the so-called false positive reactions, and with the development of the method of fixation at refrigerator temperature, to be described subsequently, it has been discarded in several laboratories. Nevertheless, the cholesterolized antigens are found, in the hands of numerous workers, to show much less variation in fixing capacity than the non-cholesterolized extracts and for this reason are recommended for routine laboratory work.

It would appear that the antigenic substance in the Wassermann test is not an antigen in the biological sense, for it can be obtained from tissues not the seat of a syphilitic infection and as has been shown by Fitzgerald and Leathes, upon injection into animals it does not lead to the formation of immune substances.

The methods of preparing syphilitic antigens have been multiplied in great number and cannot be included in the scope of this book. Simplification of preparation has been attempted with variable results. Of interest is the method suggested by Ecker and Sasano. They quote Neymann and Gager to the effect that primary extraction of the tissue with ether removes substances of anti-complementary power but only a small amount of the lecithin. Ecker and Sasano suggest three ten-minute extractions with ether in the proportion of 25. grams ground and dried heart muscle to 50. c.c. ether. The material is then extracted for one hour with 75. c.c. 95 per cent. ethyl alcohol at boiling temperature (78° C.) in a flask connected with a reflux condenser. An antigen of this sort has retained its original fixing power in this laboratory after more than a year.

Nature of Syphilitic Antigen.—Extracts of the *treponema pallidum* may serve as antigens and are true antigens in the biological sense. Craig and Nichols, however, found that alcoholic extracts of organisms closely related to *treponema pallidum*, as the *treponema pertenue* and the *treponema microdentium*, may fix complement in the presence of syphilitic serum. Extracts of animal and human

organs, particularly when prepared by alcoholic extraction appear to be distinctly more dependable than treponema extracts in the reaction of complement fixation. The exact nature of the substances in the alcoholic and in the acetone insoluble extracts is not definitely known except that lecithin constitutes a large part and that it is associated probably with other lipoids of the diaminophosphatid group, unsaturated fatty acids and certain proteins or protein fractions. That physical conditions are of importance has been known since Wassermann's early work, for it is established that a certain degree of turbidity of the antigen or its dilutions is necessary. The watery extracts are in a state of finely-suspended colloidal emulsion and, as Reudiger and others have pointed out, the dilutions of the alcoholic or acetone insoluble extracts by means of salt solution can be demonstrated to have an optimal degree of turbidity.

The Syphilitic "Amboceptor."—This is contained in the blood serum, the cerebro-spinal fluid and other juices of syphilitic patients and experimental animals. In the usual technic the blood serum is inactivated for one-half to one hour at 56° C. in order to remove complement, but in certain modifications of the test the serum is used fresh in order to utilize human complement in the reaction. Bronfenbrenner, Reudiger and others have shown that inactivation of the serum reduces its fixing power. Bronfenbrenner recommends the use of unheated serum because of the greater delicacy of the reaction. In this way it is possible to use for the test 0.04 c.c. or 0.05 c.c. serum, instead of the usual 0.1 c.c. With such small amounts of serum the human complement is a negligible factor. Long preservation or excessive heating of the serum may render it anti-lytic or anti-complementary. Contamination from unclean skin and glassware may make it either anti-lytic or lytic. The ingestion of alcohol, the presence of bile in the blood in jaundice or fat in the blood after a heavy meal or in cases of lipemia may all interfere with the activity of the fixing body in a syphilitic serum; sera in lipemia may be markedly anti-complementary. There has been much discussion of the fact that human serum may contain natural hemolysins for sheep corpuscles. In such an instance the corpuscles may be dissolved by the excess of amboceptors in spite of slight fixation of complement by the syphilitic amboceptor, thus transforming a weakly positive into a negative reaction. Sasano has found, however, that the use of an excess of immune hemolytic amboceptor, for example, ten to twenty units and one and one-half units of complement, determined by careful titration does not influence the result. Thus the factor of hemolysis by the normal anti-sheep amboceptor of human serum, which amboceptor is practically never present to the extent of more than four units, is practically negligible.

The serum may be preserved for a considerable time if kept cool and in sealed ampoules or in tightly-stoppered bottles. Reudiger has found that mixing equal parts of fresh inactivated serum and pure sterile glycerol preserves the so-called syphilitic antibody for as much as two years. Under these circumstances the sera may become anti-

complementary, but this property can be removed by heating to 56° C. for thirty minutes, and the sera are then satisfactory for use. He maintains that heated glycerolated sera give much stronger positive results than fresh unheated sera and somewhat stronger than fresh heated sera. This method is valuable for preserving known positive and negative sera as controls for the Wassermann test.

Nature of the Syphilitic Amboceptor.—The substance in the blood which acts as amboceptor is apparently closely related to the globulins, especially the euglobulin. Recently, however, Duhot has suggested that the albumin is of importance. Pfeiffer, Kober and Field, as well as Rowe, have shown an increase of globulins in syphilitic blood and spinal fluid. Noguchi has taken advantage of this fact in his butyric acid test of spinal fluid, but diseases other than syphilis may lead to increase of globulins in the spinal fluid. Peritz states that the lipid content of syphilitic serum is increased, but Bauer and Skutezky found no parallel between lipid content and Wassermann reaction. Klausner believes that the flocculent precipitate which appears on addition of 0.6 c.c. distilled water to 0.2 c.c. fresh syphilitic serum is due to the high lipid content of the serum. Weston has found no definite increase of serum cholesterol in late syphilis and no parallelism between the serum cholesterol content and the Wassermann reaction. According to Wells, "a favorite interpretation of the Wassermann reaction, which seems to harmonize with the facts, is that there is a precipitation of serum globulin by the lipoidal colloids of the antigen and adsorption of the complement by this precipitate." This is supported by the work of Jacobsthal who has demonstrated such precipitates by use of the ultra-microscope even when they are invisible to the naked eye. Holker has recently studied the colloidal phenomena and finds that the addition of antigen to syphilitic sera produces a turbidity the curve of which is steeper and higher than with normal sera. He finds that the serum is an emulsoid and the antigen a suspensoid. Salt solution disperses the serum and precipitates the antigen, thus increasing the protective state of the serum. Negative sera are much more protective than positive sera in preventing the antigen from being precipitated by salt solution.

The Complement.—As has been pointed out in the general discussion of complement, guinea-pig complement is most widely useful in immunological work. It was used by Wassermann in his original test and is extensively employed to-day. From the practical viewpoint it has certain objections. Animals are expensive and for a small number of tests it is undesirable to sacrifice an animal. This objection may be overcome by bleeding from the heart or from an ear vein (Rous), but the technic of both these operations is somewhat difficult. Owing to the lability of complement, it cannot be well preserved and the serum must be used soon after collection. The use of dried complement in filter paper has been abandoned, although such complement papers may be preserved for a few weeks in vacuum desiccators or in tubes containing calcium chloride. Drying in the frozen state in vacuo has

Table of several methods of performing Wassermann tests. From Noguchi, *Serum Diagnosis of Syphilis*.

Systems	Hemolytic system			Patient's serum	Antigen
	Complement	Amboceptor	Blood-corpuses		
Wassermann, Neisser, and Bruck.	Guinea-pig's fresh serum, known definite quantity, 0.1 c.c.	Antisheep amboceptor: that which is present normally in human serum of variable quantity, and that which is added in form of immune amboceptor (2 units).	Sheep's washed corpuscles, known definite quantity, 1 c.c. of 5 per cent. suspension.	Inactivated before use, 0.1-0.2 c.c., requiring 5 c.c. of blood.	Liquid preparation, known adequate quantity. Aqueous extract of syphilitic fetal liver.
Bauer	Do.	Antisheep amboceptor. From one source, namely in the serum to be tested. No immune amboceptor added.	Do.	Do.	Do.
Hecht	Utilizes human complement as naturally present in the fresh serum. Old specimens cannot be tested. Complement and syphilitic antibody exist inseparably in one serum if the latter is present at all. The quantity is rather variable.	As in Bauer's system.	Do.	Tested only in perfectly fresh state without inactivation. Impractical on account of inability to examine specimens several days old. Quantity definite but quite large.	Alcoholic extract of liver or heart. There is no direct way of testing its anti-complementary power on complement independent of syphilitic antibody. There is danger of obtaining non-specific reaction.
Stern	Like Hecht's system.	Like Wassermann's system.	Do.	Like Hecht's system.	Like Hecht's system.
Kaliski	Human complement 0.02 c.c. and guinea-pig's complement 0.03 c.c.	Do.	Do.	Like Noguchi's system.	Like Noguchi's system.
Detre	Rabbit's fresh serum, known definite quantity, 0.2 c.c.	Antihorse amboceptor from immunized rabbit, 2 units.	Washed horse corpuscles.	Inactivated before use. Known quantity, 0.1-0.2 c.c.	Known adequate quantity in fluid form.
Boas	Like Wassermann	Antigoat amboceptor, 2 1/4 units.	Goat corpuscles 1 c.c. 5 per cent. suspension.	Do.	Alcoholic extract of human heart.
Browning	Do.	Anti-ox amboceptor.	Ox corpuscles.	Do.	Do.
Tchernogubow	Human complement.	Anti-guinea-pig amboceptor found in patient's serum.	Washed guinea-pig's corpuscles.	To be tested while perfectly fresh.	Aqueous extract of syphilitic liver. Danger of non-specific reaction.
Foix	Do.	Anti-rabbit amboceptor found in patient's serum.	Washed rabbit's corpuscles.	Do.	Alcoholic extract of organs.

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Tchernogubow	Human complement as present in the patient's blood. Variable. The same objections as in the case of Hecht's and Stern's systems.	Antihuman amboceptor. Source unstated. An enormous quantity used, hence uneconomical.	Human corpuscles, not washed and containing fibrin ferment and fibrinogen.	Tested only while perfectly fresh, otherwise impossible. Quantity not adjustable.	Dried syphilitic liver extracted before use. Danger of nonspecific reaction.
v. Dungern . . .	Human complement contained in 0.1 c.c. of defibrinated blood and guinea-pig complement dried on paper possessing definite activity.	Antihuman amboceptor from immunized goat. Danger of missing a positive reaction on account of its source.	Human corpuscles contained in 0.1 c.c. of defibrinated blood, unwashed.	Tested only while perfectly fresh like in Tchernogubow's system. The amount of serum is that contained in 0.1 c.c. of defibrinated blood.	Alcoholic extract of guinea-pig heart. Danger of occasional nonspecific reaction.
Noguchi	Guinea-pig's serum fresh. Definite quantity (2 units), usually 0.1 c.c. of 40 per cent. dilution.	Antihuman amboceptor from immune rabbits. Liquid or dried preparations used in definite quantity (2 units).	Human corpuscles washed or unwashed, but should not contain fibrin ferment. 1 c.c. of 1 per cent., or 0.1 c.c. of 10 per cent. suspension.	Fresh or old specimens can be tested. Definite, adjustable quantities. Usually only one drop from a capillary pipette. When inactivated serum is used 4 drops are required (.08 c.c.)	Adequate quantity in liquid form or dried on paper. With inactivated serum, aqueous or alcoholic extract or pure acetone-insoluble lipoids can be used. With active serum only the pure lipoids must be used, in order to avoid nonspecific reaction.

GROUP B

been recommended by Shakell, but Karsner and Collins found that the activity was lost in eleven to fifteen days. Moledzky states that complement in the frozen states retains its strength indefinitely, but Reudiger found that although its strength is somewhat augmented at the end of one week, it deteriorates after the second week of preservation. Preservation for even these periods involves a good laboratory equipment and considerable skill. Kolmer recommends the addition of chemically pure sodium chloride to pooled guinea-pig sera in the proportion of 0.425 gram salt to 10. c.c. serum. This is effective for several weeks' preservation, and dilution is so adjusted as to restore the serum to practical isotonicity. Detre used rabbit complement, but it is not as desirable as guinea-pig complement and has the same objections. Human complement is employed in several modifications of the Wassermann test, but is present in human serum in extremely variable amounts and is difficult to titrate. It is, however, easily accessible, as it is present in the serum to be tested for syphilitic amboceptor. The selection of the complement to be used depends to a certain extent upon the hemolytic system and the modification of the test which is employed.

Complement should be used in accurately-determined amounts. Therefore, titration is of the utmost importance. Guinea-pig serum shows individual variation in complement, but in large laboratories this may be in part overcome by the "pooling" or mixing of the sera from several guinea-pigs. Such pooling, however, does not remove the necessity for titration. In some laboratories the complement is diluted 1 to 10, and the hemolytic amboceptor titrated each day by testing. We are of the opinion from experience and in view of the work of Sasano that the complement should be titrated in various dilutions (see page 190) against a constant amount of previously titrated hemolytic amboceptor. In either case the titration should take place on the same day as the Wassermann tests are made. The use of a single unit of complement does not allow for the presence of anti-lytic bodies in the reagents nor for possible deterioration of complement. At 37° C. the use of one and one-fourth units appears to be most satisfactory, whereas at ice-chest temperature the use of two units appears to be more desirable. Titration of the complement should be most accurate and the end-point be determined only by absolutely complete hemolysis.

The Hemolytic System.—Sheep erythrocytes and the corresponding hemolytic immune serum obtained from the rabbit were used by Wassermann and are widely used at the present time. Other systems include the use of goat, horse, ox, human and fowl corpuscles, with specific antisera obtained by immunizing rabbits. Certain investigators have depended upon the normal hemolysin for sheep erythrocytes often found in human serum. This is a variable quantity and almost never very large. Noguchi has summarized the hemolytic systems in a table giving all the essential data (see pages 192, 193).

The preparation of a hemolytic immune serum has been discussed (see page 117). The preservation of this serum in the moist state is

highly satisfactory if placed in amber ampoules in a refrigerator. If considered advisable preservatives may be added such as 0.5 per cent. phenol or 50 per cent. glycerol. If the serum is of high titer it may be preserved by desiccation, particularly if frozen and dried in a vacuum desiccator. Noguchi has obtained good results by drying the serum in filter paper. The filter paper is subsequently cut into strips and titrated by cutting measured lengths of the strips. We have found that this does not permit of sufficiently accurate titration and also that the titer is not well maintained.

Preservation of Erythrocytes.—If kept in a cool place without freezing, sheep erythrocytes show slight hemolysis in a few days and well-marked hemolysis in about a week. The cells of other animals show variable degrees of fragility, those of the dog being especially fragile. Various methods of preserving sheep erythrocytes for the Wassermann test have been studied by Reimann in this laboratory. The methods of particular value are preservation with formalin (Bernstein and Kaliski) and with the solutions of Rous and Turner. For formalization the sheep blood is allowed to run directly into formalin solution in the proportion of 0.5 c.c. of 40 per cent. formaldehyde solution to 400 c.c. blood. The blood is then defibrinated by shaking with glass beads, preserved in the refrigerator and before use washed three times with saline for use. The method of preservation as worked out by Rous and Turner is carried out in the following manner: The sheep is bled directly into Locke's solution containing 1 per cent. sodium citrate, in the proportion of 1 part of blood to 4 parts of solution. The corpuscles are separated by rapid centrifugalization and carefully washed three times in Locke's solution containing 0.25 per cent. gelatin. The cells are then placed in ampoules in a layer not more than 2 mm. in depth and covered with saccharose-Locke solution to a depth of about 2 cm.; the ampoules are sealed and stored at a temperature of 5° C. to 6° C. Just prior to use the cells are washed with .85 per cent. saline to remove the saccharose solution, and proper dilution effected with saline. Strict asepsis is to be observed.

THE SOLUTIONS

Locke-sodium citrate solution:

Sodium citrate	10	grams
Sodium chloride	9.2	grams
Sodium bicarb.	0.05	gram
Potassium chloride	0.1	gram
Calcium chloride	0.1	gram
Aq. dest. q.s. ad.	1000	c.c.

Locke-gelatin solution:

Gelatin	2.5	grams
Sodium chloride	9.2	grams
Sodium bicarb.	0.05	gram
Potassium chloride	0.1	gram
Calcium chloride	0.1	gram
Aq. q.s. ad.	1000	c.c.

The Locke and saccharose solutions are sterilized separately and used in the proportion of 2.8 c.c. of the saccharose solution and 7.5 c.c. of the Locke's solution.

Saccharose solution:

Saccharose	103.0	grams
Aq. q.s. ad.	1000	c.c.

Locke's solution:

Sodium chloride	9.2	grams
Sodium bicarb.	0.05	gram
Potassium chloride	0.1	gram
Calcium chloride	0.1	gram
Aq. q.s. ad.	1000	c.c.

Reimann found that the cells can be preserved for use in the Wassermann test for 3 to 4 weeks by formalization and for 21 to 25 days by the Rous and Turner method. "The readings obtained differ from those obtained with fresh cells only in so far as some sera produce slightly different results when used with cells from the same specimen of sheep blood." An excellent control for the usefulness of preserved blood is suggested by Kolmer, who maintains that there should be no discoloration of supernatant fluid after the second washing and that the blood should become brighter in color than the dark color it possesses after standing.

When extreme accuracy is desired cell emulsions are made to contain 1,000,000,000 cells per cubic centimeter. Such emulsions are being more widely adopted, but many laboratories still use 5 per cent. or 10 per cent. emulsions calculated either from the original blood volume or the bulk of the centrifuged cells. The cells should always be most carefully washed, so as to avoid precipitin reactions which may appear if the serum is not entirely removed and to wash out antilytic substances which may appear if the blood is old.

Influence of Temperature upon the Reaction.—This influence may be determined as regards the velocity of the reaction and the amount of complement fixed. The earlier work with complement fixation was based on the general assumption of immunologists that a temperature of 37° C. represents the optimum. In 1912, however, A. McNeil pointed out that ice-chest temperature favors the completeness of complement fixation in the Wassermann test, provided the time of exposure is from eight to twelve hours. This was confirmed by Coca and l'Esperance, Smith and W. J. McNeil, Berghausen and others, and the ice-chest method has now been adopted by a large number of laboratories as a standard method. The time, however, has been reduced to from three to four hours and the results appear to be entirely satisfactory. The antigen, serum to be tested and complement are mixed and placed in the ice-chest for the required time; the mixture is then brought to about 37° C. in a water bath, the sensitized erythrocytes added and the whole incubated at 37° C. for one hour.

Dean has investigated the influence of temperature and finds that fixation proceeds most rapidly at 37° C. Noguchi confirms this but finds that at the lower temperature of 23° C., fixation will reach a maximum but proceeds more slowly. He states that with the acetone insoluble antigen "a serum containing one unit of fixing substance will complete the reaction within thirty minutes at 37° C., sixty minutes at 30° C., and two hours at 23° C., irrespective of whether human or guinea-pig complement is used." Dean, however, finds that at 0° C. the amount of complement fixed is much greater than at 37° C., and this accords with experience in the use of the ice-chest method. Certain unknown factors may delay the action of the complement, as has been pointed out by McConnell, and a second incubation may accordingly have to be prolonged beyond the usual time.

The Technic of the Wassermann Test.—For the demonstration of the method we may use an alcoholic extract of ox heart as the syphilitic antigen, inactivated human serum from a normal individual and from a known victim of syphilis, guinea-pig complement and a sheep hemolytic system.

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The antigen may be made by weighing 10 grams ox heart which has been freed from blood, fat and connective tissue, ground in a meat grinder and dried under a current of air from an electric fan or in a desiccator. It is then extracted in 100 c.c. 95 per cent. ethyl alcohol, first by shaking 18 hours in an electrical shaker and then standing for 5 days at 37° C. It is filtered and kept tightly stoppered in an amber glass bottle in the refrigerator. For use, slowly add 9.0 c.c. physiologic salt solution to 1.0 c.c. alcoholic extract. This constitutes the "antigen dilution" of the charts. It must then be titrated to determine its antilytic properties as well as its lytic powers. The following tests of the antigen may be set up after previously determining the titer of the hemolytic amboceptor and complement. In the following titrations the complement is diluted so that 1.0 c.c. contains 1 unit, the amboceptor so that 1.0 c.c. contains 2 units. In the first series, volume is made up by addition of salt solution, so that each tube contains 4.0 c.c. and in the second series so that each tube contains 2.0 c.c.

TITRATION OF ANTIGEN FOR ANTILYTIC PROPERTIES

Antigen dilution	Complement	Incubate one hour	Hemolysin	5 per cent. cell suspension	Incubate one hour	Result
1.0 c.c.	1 unit		2 units	1 c.c.		P.H.
0.8 c.c.	1 unit		2 units	1 c.c.		P.H.
0.6 c.c.	1 unit		2 units	1 c.c.		P.H.
0.4 c.c.	1 unit		2 units	1 c.c.		C.H.
0.2 c.c.	1 unit		2 units	1 c.c.		C.H.
.....	1 unit		2 units	1 c.c.		C.H.

TITRATION OF ANTIGEN FOR LYTIC PROPERTIES

Antigen dilution	5 per cent. cell suspension	Incubate one hour	Result
1.0 c.c.	1 c.c.		P.H.
0.8 c.c.	1 c.c.		P.H.
0.6 c.c.	1 c.c.		—
0.4 c.c.	1 c.c.		—
0.2 c.c.	1 c.c.		—
.....	1 c.c.		—

In the protocols P.H. indicates partial hemolysis, C.H. complete hemolysis and (—) no hemolysis. Thus it is seen that 0.6 c.c. is the smallest amount of antigen which is antilytic and 0.4 c.c. the largest amount which is not. For practical purposes one-half the latter amount, or 0.2 c.c., is the largest amount which may be used. This is considerably smaller than the amount of antigen which possesses hemolytic properties in itself, as shown in the second protocol.

After obtaining this information, the antigen should be titrated to determine the smallest dose that fixes complement in the presence of a known syphilitic serum. A strong serum (++++) may be obtained from a laboratory or if not available a serum may be secured from a patient in the florid secondary stage of the disease. This serum is used in constant amounts of 0.2 c.c. More delicate titration is accomplished by the use of a known ++ serum either alone or in addition to the ++++ serum. Knowing that 0.2 c.c. is the largest dose of antigen dilution that may be employed the test is set up with that as the maximum amount of antigen, followed by decreasing doses. (See table on page 198.)

The protocol includes the necessary controls, showing that neither antigen (12), syphilitic serum (7), nor non-syphilitic serum (19) exhibits antilytic powers. It shows that antigen (13), syphilitic serum (15) and non-syphilitic serum produce no hemolysis. It shows that non-syphilitic human serum (15-18) fails in the presence of the antigen to fix complement. It shows that in the presence of syphilitic serum the antigen solution in amounts as small as 0.01 c.c. fixes complement. That amount, 0.01 c.c., is the fixing dose and is doubled for the actual Wassermann test.

TITRATION OF ANTIGEN FOR FIXING PROPERTY.

Tube	Antigen dilution	Syphilitic serum	Complement		Hemolysin	5% Cell suspension		Result
1	0.2 c.c.	0.2 c.c.	2 units	Incubate one hour	2 units	1 c.c.	Incubate one hour	—
2	0.1 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		—
3	0.05 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		—
4	0.01 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		—
5	0.005 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		P. H.
6	0.001 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
7	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
8	2 units		2 units	1 c.c.		P. H.
9		2 units	1 c.c.		—
10	1 c.c.		—
11	2 units		1 c.c.		—
12	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
13	0.2 c.c.	1 c.c.		—
14	0.2 c.c.	1 c.c.		—
		Non-syphilitic human serum						
15	0.2 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
16	0.1 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
17	0.05 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
18	0.01 c.c.	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
19	0.2 c.c.	2 units		2 units	1 c.c.		C. H.
20	0.2 c.c.	1 c.c.		—

Other Reagents for the Test.—The methods of obtaining guinea-pig blood, the hemolytic amboceptor and sheep blood have been described (see pages 117 and 127). Various methods are in vogue for obtaining human blood. In adults the simplest satisfactory method is to obtain the blood by puncture of one of the large veins in the cubital fossa anterior to the elbow-joint. The needle should have a calibre of about 1. m.m. and although sharp should not have an elongated point. The fossa is cleansed with soap and water followed by alcohol. A tourniquet is applied at the middle of the upper arm and the patient instructed to "make a fist" several times until the veins stand out prominently. The sterile needle is inserted and the blood collected in amounts of 5 to 10 c.c. in a 15 c.c. centrifuge tube. The tourniquet is released before the needle is withdrawn and the wound sealed with collodion. The blood is allowed to clot, the clot separated from the side of the tube by means of a sterile needle, and allowed to contract for several hours in the refrigerator. The tube is then centrifuged and the serum pipetted into another tube so as to avoid hemolysis. The serum is inactivated at 56° to 60° C. for one-half hour before testing, unless the test is to be made by a modification which employs human complement. Methods have been suggested in which the amount of blood obtained by puncture of finger tip or ear lobe provides sufficient blood. In infants or obese adults blood may be obtained by the use of a scarifier and cupping. Bleeding from the longitudinal sinus, from the great toe and from the heel are also practised in infants.

The Test.—With the reagents at hand the test is set up with one or more antigens. In many laboratories different types of antigen are employed, as for example an acetone insoluble antigen, a cholesterolized alcoholic extract of heart muscle and a non-cholesterolized alcoholic extract of heart muscle. Others are employed as the operator sees fit. Antigens may deteriorate, so that it is wise to have several on hand and under observation in the test. The protocol shows 2 antigens of the same strength. All the elements in the test are to be controlled to prove that they are not antilytic and to show that the hemolytic system operates properly. In addition it is essential to have controls with a known positive and a known negative serum. The antigen, complement and hemolysins are diluted so that the proper quantity of each is contained in 1.0 c.c. It appears to be desirable to add the human serum without dilution. This is done with a 1.0 c.c. pipette graduated in hundredths of a cubic centimeter. The dotted lines in the body of the protocol indicate that salt solution is to be substituted in quantities of 1.0 c.c.

THE WASSERMANN TEST

Tube	Antigen No. 1 dilution	Antigen No. 2 dilution	Human serum	Comple- ment		Hemolysin	5 % cell suspension	Result
1	0.02	0.2	2 units	Incubate one hour	2 units	1 c.c.	P. H.
2	0.02	0.1	2 units		2 units	1 c.c.	
3	0.02	0.2	2 units		2 units	1 c.c.	
4	0.02	0.1	2 units		2 units	1 c.c.	
			Known positive					P. H.
5	0.02	0.2	2 units		2 units	1 c.c.	
6	0.02	0.1	2 units		2 units	1 c.c.	
7	0.2	0.2	2 units		2 units	1 c.c.	
8	0.2	0.1	2 units		2 units	1 c.c.	
			Known negative					C. H.
9	0.02	0.2	2 units		2 units	1 c.c.	
10	0.02	0.1	2 units		2 units	1 c.c.	
11	0.02	0.2	2 units		2 units	1 c.c.	
12	0.02	0.1	2 units		2 units	1 c.c.	
13	0.02	2 units		2 units	1 c.c.	C. H.
14	0.02	...	2 units		2 units	1 c.c.	
15	0.2 (test serum)	2 units		2 units	1 c.c.	
16	0.2 (positive)	2 units		2 units	1 c.c.	
17	0.2 (negative)	2 units	Incubate one hour	2 units	1 c.c.	C. H.
18	2 units		2 units	1 c.c.	C. H.
19		2 units	1 c.c.	—
20	1 c.c.	—

The results of the Wassermann test are usually indicated by plus signs; the following diagram indicates the interpretation of the results:

DEGREE OF HEMOLYSIS		
WITH		
0.2 c.c. human serum	0.1 c.c. human serum	Result
—	—	++++
—	P. H.	+++
—	C. H.	++
P. H.	C. H.	+

In these readings the partial hemolysis is relatively small in amount. If with 0.2 c.c. human serum the hemolysis is well advanced without being complete and is complete with 0.1 c.c. serum, the result is indicated by the sign +. Other symbols are used, but the results are indicated in the same general way.

Reference to the protocol shows that the serum in tubes 1, 2, 3, 4 is positive for syphilis and would be signified as a three plus (+++) serum. The known positive is a four plus (++++) and the known negative reacts properly. Tubes 13 and 14 show that the antigens are not antilytic, and tubes 16, 17, 18 show that the sera are not antilytic. Tube 18 shows that the hemolytic system operates properly. Tube 19 shows that the hemolysin does not produce hemolysis without complement, and tube 20 shows that the corpuscles do not hemolyze without the other agents.

The quantities given in the protocol are based on a unit of 1.0 c.c. to simplify the explanation. In order to save reagents the quantities are usually divided in half, so as to be on a 0.5 c.c. basis. The directions for the United States Army in France called for quarter quantities, so as to save reagents. The latter directions also call for half saturation of the alcoholic heart extract with cholesterol (0.2 per cent.). Bronfenbrenner has suggested the use of 0.1 c.c. amounts of the reagents. Methods of measuring by drops have been employed, but are inaccurate because of the possible variation in the size of drops unless a stalagmometer or similar instrument is employed.

Modifications of the Tests.—Numerous modifications of the test have been recommended. These are based on variations in syphilitic antigen, various ways of treating the human serum, differences in selection of the complement and in selection of the hemolytic system. These are indicated in the chart on page 192. It is our opinion that any method, to be acceptable, must permit of accurate measurement of the reacting bodies. The possibilities as to methods of preparing antigen and human serum have been discussed. The use of human complement in the test interposes errors, which we believe have not been overcome. The titration of human complement must differ with different specimens and in the Gradwohl method fails to take account of the variable content of natural hemolytic amboceptor in human serum. Of the hemolytic systems recommended, the most satisfactory are the sheep or goat and the human systems. In most laboratories the sheep system appears to be most accessible and the factor of error introduced by the presence of normal anti-sheep amboceptors in human serum can usually be overcome by absorption with sheep erythrocytes or can be controlled by the use of one and one-half units of complement. The human hemolytic system largely obviates this objection, but it is sometimes difficult to obtain enough blood to immunize animals for the production of the specific immune hemolysin. We also suggest the possibility that an unusually strong natural iso-hemolysin in the tested serum may confuse the results. Kolmer, in a recent study, has found that the human hemolytic system considerably increases the delicacy of the reaction, especially when small amounts of the patients' sera are employed. In positive cases he found 10 per cent. more positive reactions by the use of the human system than with the sheep system.

The Specificity of the Wassermann Reaction.—Numerous studies have been made as to the specificity of the test in the different stages of syphilis. In evaluating such figures certain factors of error in the actual performance of the test must be considered. Unless the worker is familiar with the many factors which may influence the reaction of hemolysis and the fixation of complement, as pointed out briefly in the chapter on hemolysis and the discussion of complement fixation, the results may be misleading. The type of antigen employed is also of significance as influencing the results. Of no small importance is the operator himself, for although the Wassermann test may properly be regarded as a physico-chemical test rather than a strictly biological or immunological reaction, nevertheless it requires a thorough understanding of immunological procedures. Tests made in the hands of persons trained to perform this test, without broader training, are not to be given the same value as tests in the hands of broadly-trained immunologists. The subject of specificity of the test is closely bound with the clinic, in which certain factors of error in clinical diagnosis must be accepted. Until more satisfactory methods are provided for in the post-mortem room, the factor of error there is almost as large as in the clinic. Warthin, by particularly refined methods applied to

cases which have been examined shortly after death, has shown the presence of the treponema in lesions which previously had not been positively known to be syphilitic. Symmers, Darlington and Bittman found a considerable divergence between ante-mortem Wassermann tests and the post-mortem evidence of syphilis, but Turnbull finds a striking agreement. Certainly syphilis can progress for a long time without gross morbid anatomical manifestations, and it seems possible that the pathologist cannot be sure of excluding syphilis in his anatomical diagnosis. Improved technic is the only way of reducing this factor of error and thereby providing an accurate control of the Wassermann and other clinical tests.

The Diagnostic Value of the Wassermann Test.—Naturally this subject has been studied extensively and figures vary as the technic is improved. In 1914 Boas published an analysis of over 8000 cases reported in the older literature and tabulates them as follows:

	Number of cases	Positive	Per cent. positive
Primary syphilis	1060	629	59
Secondary syphilis	3526	3181	90
Tertiary syphilis	1212	1020	84.1
Early latent syphilis	983	504	51
Late latent syphilis	1520	605	39
Tabes dorsalis	159	115	72
Paresis	405	402	99.2

These figures are sufficient to indicate that the Wassermann test is of distinct value in the diagnosis of syphilis. More recent statistics offered by Craig as the result of tests carried out by himself illustrate the accuracy of the reaction as applied under excellent conditions. In interpreting the following figures from Craig, given as the result of a single test on each of 4658 cases diagnosed as syphilis, it must be remembered that there is at least a small factor of error in the clinical diagnosis. The table follows:

	Number of cases	Positive	Per cent. positive
Primary syphilis	908	813	89.5
Secondary syphilis	1889	1817	96.1
Tertiary syphilis	638	558	87.4
Latent syphilis	1173	790	67.3
Congenital syphilis	28	25	89.2
Parasyphilis	22	7	68.1
	4658	4010	86.2

Tests made by Craig on 2643 individuals, either not diseased or victims of disease other than syphilis, showed the reaction to be positive in eleven instances (0.4 per cent.). These eleven instances included four cases of malaria, three of tuberculosis (two of which ultimately gave a clinical history of syphilis), three cases of pityriasis rosea and one case in which the diagnosis was not established. It is to be considered possible that diseases other than syphilis may produce those changes in the blood which lead to fixation of syphilitic antigen and complement; among these are occasional cases of leprosy, scarlatina, malaria, try-

panosomiasis and certain skin diseases. Gordon, Thomson and Mills have recently insisted that malaria will not produce a positive reaction unless complicated by syphilis or as the result of faulty technic. Although a controversial point, we believe that occasional cases of tuberculosis may give a positive Wassermann test. That this is not necessarily due to coincident infection with syphilis is shown by the experience of Petroff, who found a positive Wassermann in a tuberculous cow.

Interpretation of Results.—Craig and others are of the opinion that a strongly positive result, such as would be indicated by ++++ in our schedule, is conclusive evidence of syphilis, whether there are symptoms or not. Other degrees of fixation must be interpreted with the aid of clinical history and symptoms. A single negative reaction does not exclude syphilis. In doubtful cases the so-called provocative treatment should be applied. This means that a short course of mercury or preferably half the usual dose of salvarsan or neosalvarsan should be given and the Wassermann test made subsequently. It is advisable to test the blood twelve, and twenty-four hours after provocative administration of salvarsan as well as every day for at least ten days. If the reaction is to become positive, it usually does so in from a few hours to five or six days, but may be delayed for ten days or even more. That this is an absolutely specific effect of the drug is contradicted by the report of Wildgren, who found that the injection of milk may produce similar results. Endless discussion might be presented as to the interpretation of the Wassermann test in the clinical diagnosis of syphilis, but we incline to the view that this test, as is true of many laboratory examinations, is to be regarded as important evidence in clinical diagnosis, is of striking specificity when properly performed, but is not absolutely pathognomonic.

Dependability of the Test.—Criticism has been directed against the test because of the fact that results do not always agree with clinical findings and because of differences in results upon the same serum in different laboratories. It must be admitted that the factors of error in the test are greater than in clinical diagnosis of the disease. Discrepancies in reports from different laboratories may, in part, be due to inherent faults in the test, to faults in technic, to faults in selection of materials and to insufficient training of the worker. The older literature contains serious criticisms of the test, as for example the papers of Wolbart and of Uhle and Mackinney. Under the direction of the Medical Research Committee of Great Britain in 1918 the results obtained independently by Dr. C. H. Browning, Dr. J. McIntosh and Col. L. W. Harrison upon the same specimens are in very close agreement. More recently Solomon has analyzed the results of 3000 tests carried out in two different laboratories by skilled workers, Dr. Hinton and Dr. Castleman. There was complete agreement of results in 93.44 per cent. of this large series of tests. This study demonstrates that with modern methods and skillful performance of the test results are highly dependable.

Quantitative Results with the Wassermann Test.—For various purposes, more particularly the observation of the results of treatment, it may be desirable to titrate accurately the amount of patient's serum which serves as an amboceptor. This may be done by using different quantities of the serum. Dilutions of the serum are made with salt solution, 1 to 4, 1 to 8, 1 to 16, 1 to 32, 1 to 64, or are measured as 0.1 c.c., 0.05 c.c., 0.03 c.c., 0.02 c.c., 0.01 c.c., etc. The tubes are treated in the usual fashion and the results recorded as $\frac{F}{8}$, indicating complete fixation in dilution 1 to 8, $\frac{P}{16}$ indicating partial fixation in dilutions of 1 to 16, $\frac{H}{32}$ indicating hemolysis or no fixation in dilutions of 1 to 32.

Wassermann Test on Spinal Fluid.—Spinal fluids are not inactivated and are employed in larger volumes than blood serum, up to as much as 1.0 c.c. Hauptmann and Hossli were the first to insist upon the use of large quantities of spinal fluid, and this modification changed the entire conception of the frequency of positive results in the spinal fluid of such diseases as paresis and tabes dorsalis. The test with spinal fluid is of particular value in syphilis of the central nervous system, where it is somewhat more specific than the test with blood serum. The test has also been used with success with transudates and exudates from the peritoneum, pleura and pericardium. Apparently of value in examination of the spinal fluid is the Lange colloidal gold test, described in texts of clinical pathology.

Post-mortem Wassermann Tests.—In a certain number of cases, death ensues too soon after the patient comes under observation to secure blood for the Wassermann test. Not infrequently the result of a Wassermann test may aid the pathologist in morbid anatomical diagnosis and may furnish information of value to the clinician in the consideration of doubtful cases. The question arises as to whether or not post-mortem changes in the blood will invalidate the Wassermann test. Valuable information has been collected by Graves. In a series of 400 cases he found that only 0.46 per cent. of sera from cadavers were antilytic and only 0.58 per cent. of sera were hemolyzed, coagulated or otherwise unfit for use. The post-mortem and ante-mortem results were the same in 97 per cent. of sixty-eight controlled cases. "The reactions conformed to the anatomic and historical evidence in 304 of 378 cases, or 80.4 per cent." Contradictory findings are recorded in less recent literature, but we believe that valuable results may be obtained with blood taken after death.

COMPLEMENT FIXATION IN TUBERCULOSIS

The advantage of a complement-fixation test in the diagnosis of early pulmonary tuberculosis and in concealed or suspicious lesions is obvious. Certain authors, Craig, Miller, von Wedel, report a high percentage of positive reactions in tuberculous individuals, whereas others, Cooper and Lange, report relatively few positive results. Petroff is of the opinion that these differences may be due to lack of complete

and careful study of the cases clinically, as well as a failure to observe minute details of the test.

Antigens are of the utmost importance, and numerous forms have been suggested. There appears to be well-founded evidence for using several strains of the human type bacillus associated with one or more strains of bovine type. The methods of making antigen vary and include the use of saline suspensions or extracts of tubercle bacilli, living or dead, intact or pulverized; filtrates of broth cultures; ether alcohol extracts of whole or autolyzed bacilli, and extracts of tuberculous organs. Apparently those extracts which contain both lipoids and proteins are most satisfactory. The antigenic substance is thermostable.

The human serum is inactivated, and in Petroff's hands appears to be most satisfactory if collected one or two days before the test. Accurate titration of complement, to be used in doses of two units, and of hemolytic amboceptor is essential. Guinea-pig complement and a sheep-rabbit hemolytic system are satisfactory. It is absolutely essential that glassware be perfectly clean and that measurements be accurate. The incubation of the mixture of antigen, tuberculous amboceptor and complement should be from one and one-half to two hours at the optimal temperature of 35°-40°.

Wilson, using a lipid-free bacillary antigen, attaches great importance to the complement and finds that there is not a universal adaptability of guinea-pig complement. That from some guinea-pigs appears to be fixed more readily than that from others. Therefore, in general, pooled complements are likely to give the best results. If a single complement is used tests should be made to determine the extent of fixation. Von Wedel states that preservation of the patient's serum in the ice-box for five to seven days favors the reaction, but Petroff found that fresher sera are preferable. It is desirable to make several tests at intervals upon the same patient. As the result of 1555 tests on 713 cases Petroff obtained the following results:

	Cases	Positive	Negative	Percent.
Clinically active tuberculous	212	199	13	93.9
Quiescent tuberculous	158	89	69	56.3
Apparently cured more than two years	58	5	53	8.5
Normals	78	3	75	3.8
Suspected	166	65	101	39.1
Other diseases	41	6	35	14.6

An analysis of these figures shows that under proper conditions complement fixation is of distinct value in the diagnosis and prognosis of tuberculosis. Basing his conclusions on experimental data Petroff considers "the complement-fixation test in tuberculosis more specific than the Wassermann test" in syphilis, an opinion in which we concur. Nevertheless, its most ardent advocates do not regard the test as pathognomonic and Petroff regards it as "only one of the many links in the tuberculosis diagnostic chain." It is unfortunate that the complement-fixation test gives the highest percentage of positive results in cases in which the need for such diagnostic aid is least evident, namely in those

cases of active tuberculosis in which the diagnosis on clinical and bacteriological grounds is reasonably certain.

"Acid-Fast Fixation."—Of great interest is the fact as shown by Cooke and others that the complement-fixation test affirms the close biological relationship of the acid-fast bacilli. From rabbits immunized with various acid-fast bacilli Cooke obtained sera which reacted interchangeably with each member of the group employed in the experiment. In certain instances the immune sera reacted somewhat more strongly with their own antigenic organism than with others of the group. Cooke also found that the sera of tuberculous patients react not only with the tubercle bacillus but also with other acid-fast bacteria. Sera from cases of leprosy also contain complement-binding substances which react with antigens made from several members of the acid-fast group, the cases of nodular leprosy giving more striking fixation than those of anesthetic type. According to Cooke, the Wassermann test gives crossed reactions in tuberculosis which are too frequent to be explained by the coincidence of syphilis.

COMPLEMENT FIXATION IN GONOCOCCUS INFECTIONS

As with other immune reactions of the animal body, time plays an important part in the production of complement-fixing bodies in gonococcal infections. Acute gonorrhea is usually diagnosed with ease by bacteriological methods, but it is not until the disease has persisted several weeks that complement-fixing bodies are likely to be demonstrated. The value of complement fixation appears in those cases where simpler bacteriological methods are not adaptable, such as gonorrheal rheumatism and endocarditis, as well as infections of deeper parts of the genital tract, such as the Fallopian tube, Cowper's glands and prostate. The test is also useful in determining the cure of the disease.

Müller and Oppenheim in 1906 reported favorable results with the gonococcus complement-fixation test. Bruck and subsequently Meakins had a similar experience, but more recent study indicates that the older methods possess little specificity. The work of Teague and Torrey, Wollstein, Watabiki and Schwartz and McNeil demonstrated the occurrence of numerous immunologically distinct forms of gonococcus and the necessity for using several strains in the antigen. It now appears that from ten to fourteen strains are desirable.

The production of antigen has been extensively studied. Salt solution extracts appear to be satisfactory. Alcohol extracts have no value, and Wilson believes that a lipoid-free antigen presents an improvement in titer, stability and freedom from anti-complementary activity. Warden claims good results with an antigen composed of salts of the fats of the gonococci. Thomson, working under the direction of Col. L. W. Harrison, reports excellent results by dissolving the organisms in decinormal sodium hydrate solution and restoring to the neutral point by decinormal hydrochloric acid. In the hands of most workers the sheep-rabbit hemolytic system appears to be satis-

factory, but it has the same objections as obtain in the Wassermann test. A human-rabbit system may be substituted if desired.

The test appears to be highly specific and of great clinical value when properly performed. Although the gonococcus and meningococcus are closely-related organisms and may, according to Wollstein, give crossed complement-fixation reactions there is no satisfactory evidence that epidemic cerebrospinal meningitis in man produces confusing complement-fixing bodies. Dixon has recently studied 840 tests made by Dixon and Priestly on 625 individuals. Of fifty-three strongly positive reactions 90.4 per cent. had gonorrhea or a history of the disease, of sixty-six moderately strong reactions 86.3 per cent. were confirmed clinically, of seventy-five weakly positive reactions 72 per cent. were confirmed clinically, of ninety doubtful reactions 58.9 per cent. were clinically cases of gonorrhea. Of 341 negative reactions 26.1 per cent. were cases of gonorrhea in some form; of these only one case was positive to a second test. Therefore, a positive test is to be regarded as strong presumptive evidence of the disease, but both positive and negative reactions should be controlled by subsequent tests.

OTHER COMPLEMENT-FIXATION TESTS

Glanders.—The complement-fixation test in this disease appears to be highly specific independently of the strain of the antigenic organism. Its principal application is in the disease as it affects horses. The mallein test and the agglutination test are satisfactory but can be supplemented by complement fixation. Occasionally it may be serviceable in human medicine.

Typhoid Fever.—Although earlier workers obtained variable results, later investigations in the hands of Garbat and of Kolmer with salt solution extracts of numerous strains of the bacillus, the so-called polyvalent antigen, have given excellent results more particularly in the second or third week of the disease or later. Blood cultures, the Widal and the Dreyer tests are so much more easily performed that the complement-fixation test is to be regarded as only supplementary. Nevertheless, complement fixation is more likely to occur in the course of the disease than as the result of prophylactic vaccination and accordingly may gain diagnostic value.

Smallpox.—Positive results have been obtained in this disease by Jobling, Sugai, Dalm, Klein, Kolmer and others. The antigen has been obtained either from the lesions of vaccinia in calves or from human smallpox lesions. Salt solution extracts appear to be better than alcoholic extracts. In addition to the diagnostic value, the reaction adds to the evidence concerning the biological identity of smallpox (*variola*) varioloid and vaccinia. Our interpretation of Xylander's results indicates that vaccinia in man does not lead to the establishment of complement-fixing bodies over a long period of time and therefore in all probability is not a true index of immunity to smallpox. The great diagnostic

value lies in the differentiation of smallpox from syphilis and from chicken-pox (varicella).

Whooping-Cough.—With antigens made from the pertussis bacillus of Bordet-Gengou the reaction appears to have considerable diagnostic value.

Echinococcus Cyst.—The antigen is obtained by filtering the cyst fluid of man or sheep and preserving with 0.5 per cent. phenol in a cool place. Varying results have been reported, but the test appears to be worthy of further investigation where material can be obtained for its use.

Malignant Tumors.—Numerous attempts have been made to aid in the diagnosis of malignant tumors by the complement-fixation test, using antigens prepared from tumor material. The results have been conflicting. Von Dungern has devised a test using, on empirical grounds, an antigen prepared by making an acetone extract of normal human red blood-cells. He has obtained fixation in as high as 90 per cent. of known cases of malignant tumors. The test, however, has not as yet been sufficiently widely applied to justify recommending it as of clinical value.

Sporotrichosis.—Widal, Abrami, Joltrain and Weil have obtained excellent results using as antigen the sporotrichum *Beurmanni*. Moore and Davis have recently demonstrated fixation with a human serum in the presence of Schenck-Hektoen, Beurmann and Davis strains of the organism. This reaction, in addition to agglutination, is of distinct diagnostic value.

CHAPTER X

HYPERSUSCEPTIBILITY

DEFINITION.

OCCURRENCE.

ANAPHYLAXIS.

SENSITIZATION.

PERIOD OF INCUBATION.

INTOXICATING INJECTION.

THE REACTION.

CLINICAL PHENOMENA.

DISTENTION OF LUNGS.

FALL IN BLOOD-PRESSURE.

METABOLIC AND BLOOD CHANGES.

DECREASED COAGULABILITY OF BLOOD.

DESENSITIZATION.

PASSIVE ANAPHYLAXIS.

SPECIFICITY OF ANAPHYLAXIS.

THEORIES OF REACTION.

ANAPHYLACTIC POISONS.

CELLULAR THEORIES.

PHYSICAL THEORIES.

ANAPHYLACTOID PHENOMENA.

SUMMARY.

THE RELATION OF ANAPHYLAXIS TO IMMUNITY.

Definition.—On casual consideration hypersusceptibility appears to be a condition exactly the opposite of immunity. If by immunization an animal becomes more than normally resistant to a poisonous or infective agent so in the state of hypersusceptibility it is more than normally susceptible to poisons, to infective agents and to agents which in the normal animal appear to be entirely innocuous. More critical examination of the phenomenon, however, has led to the conception that hypersusceptibility is but one manifestation of the intricate mechanism of immunity. The reasons for this latter conception will appear in the subsequent discussion. The term hypersusceptibility is not to be confused with anaphylaxis, with which, in our judgment, it is not synonymous. We prefer to limit the term anaphylaxis to that state of hypersusceptibility to a given substance which has been induced by a previous injection of the same substance. The reaction is limited to proteins or protein fractions. Natural hypersusceptibility to non-protein substances may occur, but this condition cannot be induced by a previous administration of such substances.

Occurrence.—Hypersusceptibility may be natural or acquired. Undoubtedly certain individuals in whom the condition is supposed to be natural have acquired the state by preliminary inoculation of the substance to which they are susceptible. This may be an unconscious, forgotten or concealed acquisition. The introduction of practically any protein into the tissues of the body may lead to the acquisition of a hypersusceptibility of long duration unless the primary inoculation is succeeded by others at proper intervals and in proper amounts to produce

immunity. In man natural hypersusceptibilities are believed to be manifested upon the introduction of the special proteins or similar substances into the respiratory tract, the alimentary canal, into the skin and by injection into the tissues, body spaces or circulation. Man may exhibit respiratory symptoms in the presence of vegetable effluvia, as in "hay fever," "rose fever," and of the effluvia of certain animals, such as the horse and guinea-pig. In individuals thus susceptible, local or general reactions may occur following inoculation with the specific animal or vegetable protein. The ingestion of animal proteins, such as egg, or vegetable proteins, such as strawberry, may produce severe gastro-intestinal disturbances sometimes accompanied by general symptoms. In certain cases this hypersusceptibility may have been acquired by previous sensitization, but in the greater number no such explanation is to be offered. In babies susceptible to egg-white there is no probability that preliminary direct sensitization occurs, but it is possible that the tendency to hypersusceptibility may have been inherited. The instances mentioned are examples of individual hypersusceptibility. Although less clear cut there are also evidences of species hypersusceptibility, as, for example, the fact that ox serum is distinctly toxic for guinea-pigs and much less so for man. The acquired forms of hypersusceptibility will be considered under the general discussion of anaphylaxis.

Anaphylaxis.—Following the introduction of the serum treatment of disease, disturbing elements appeared, the most striking of which were the frequent production of "serum rashes" and the reports of occasional severe constitutional reactions and even sudden death. Von Pirquet and Schick pointed out on the basis of a clinical investigation in connection with the serum treatment of diphtheria and scarlatina, that in from seven to twelve days following a single injection of serum or several injections on successive days, a so-called "serum disease" appears. This is characterized by macular or maculo-papular eruptions of urticarial type, malaise, fever and other symptoms. After this period a subsequent injection of the same protein leads to the appearance of similar symptoms and signs usually within twenty-four hours. After the lapse of months or years the reaction may be delayed and fail to appear for several days, but is only rarely as late as that following the primary injection. In other words, the patients appeared to have been sensitized by the primary injection. Not being able to define exactly the nature of this condition, the name *allergy* was suggested, indicating an "altered state" of the animal body. The usage of the term at the present time is confusing and definitions vary; we, therefore, prefer not to employ it.

Experimentally similar phenomena had been noted in the course of other studies as far back as Magendie in 1839, but it remained for Richet and Portier in 1902 to point out the fact that an animal may be rendered hypersusceptible to a poison, by the previous injection of a small dose. They used actino-congestine, a toxic protein extracted from the tentacles of actinia. Because the phenomenon indicates a

condition the opposite of prophylaxis they named it anaphylaxis. As a result of the reports of accidents following the use of diphtheria antitoxin, Rosenau and Anderson investigated the problem experimentally and found that the danger lies in the serum rather than in its content of antitoxin. They demonstrated that the reaction is specific for the protein employed, that the period of "incubation" is about six days and that once established the sensitive state persists for many months with but slight reduction in intensity. In the same year, 1906, Otto entirely independently published similar findings in Ehrlich's laboratory as the result of an interview between Ehrlich and Theobald Smith. Smith had noted that animals used for the titration of diphtheria antitoxin were subsequently extremely sensitive to horse serum. Otto, accordingly, employed the name Theobald Smith phenomenon. Of somewhat similar significance, but for the time without the same direct application to medicine, were the investigations of Arthus, who in 1903 published a study in which he showed that if repeated subcutaneous injections of protein are given, the fourth and subsequent injections may lead to severe local reactions which may go on to gangrene. If a later injection is given intravenously death may result. Arthus also recognized the specificity of the reaction. The year 1906 marked the beginning of a period of widespread investigation of anaphylaxis. Much has been learned in regard to the mechanism of the process, but the fundamental principles are still in the form of hypotheses.

The Sensitization.—The substances necessary for the demonstration of anaphylaxis are proteins. These need not contain all the amino-acids, for Wells has shown that certain vegetable proteins, zein, hordein, gliadin, lacking "one or more such amino-acids as glycocholl, tryptophane or leucine produce typical reactions" and Abderhalden claims to have demonstrated anaphylaxis with a compound polypeptid made up of fourteen amino-acid molecules, which include only two of the amino-acids, leucine and glycocholl. The sensitizing substance is extremely thermo-resistant. Wells finds that proteins such as casein and ovo-mucin which are not heat coagulable are active after heating to 100° C. and Besredka has found that if a coagulable protein is so diluted as to prevent coagulation it withstands temperatures up to 120° C. Rosenau and Anderson found that if the protein be in the dry state it may be heated to 170° C. for ten minutes and upon re-solution will serve for the production of anaphylaxis. Heat or chemical agents which render the protein insoluble destroy its sensitizing properties. Trypsin digestion has the same effect. Gay and Adler reported that upon fractioning serum with ammonium sulphate the euglobulin contains the sensitizing substance, but not that substance which intoxicates at the second injection. Kato, however, finds that the globulins possess the largest content of both sensitizing and intoxicating substances. Bogolomez and subsequently Meyer claimed that anaphylaxis could be produced with lipoids but this has failed of confirmation in the hands of Wilson and of White and others; it is not generally accepted. The chief dif-

ficulty in the work with lipoids lies in the fact that it is practically impossible to obtain the lipoids in pure form; an extremely small amount of adsorbed protein may produce the reaction.

The method of sensitization is by parenteral routes, although Rosenau and Anderson in their original communication state that they had been able to sensitize guinea-pigs by feeding horse serum. Besredka was unable to confirm this, but in a few dogs we have obtained results which have been highly suggestive. A question of fundamental importance in this connection is whether or not proteins may be absorbed through an intact intestinal mucosa without digestion. According to the work of Van Alstyne and Grant, such absorption may occur. Absorption of the whole protein through the intestinal mucosa or the mucosa of other surfaces might well serve to sensitize animals or man, but as yet the problem is not conclusively settled. If the inoculation be by parenteral routes there is apparently little difference in outcome whether administered subcutaneously, intravenously or intraperitoneally.

The amount of protein necessary for sensitization is extremely small. In their original work, Rosenau and Anderson found that in guinea-pigs 0.000,001 c.c. horse serum suffices. Wells succeeded in sensitizing guinea-pigs with 0.000,000,05 gram crystallized egg albumin. Larger sensitizing doses are necessary in order to produce subsequent death from anaphylactic shock. Such minute doses are not applicable in the case of rabbits, dogs and monkeys, in which it may be necessary to inject the material on two or three successive days in order to sensitize. The minimal sensitizing dose in man is not known. In experimental animals there is an optimal sensitizing dose which bears a certain relation to the subsequent intoxicating dose, as has been shown by White and Avery. In a general way, the smaller the sensitizing dose, the larger the minimum intoxicating dose and vice versa, but a sensitizing dose may be too large for satisfactory sensitization. According to Besredka the larger doses also require a longer time for sensitization to appear.

Period of Incubation.—For a period of eight to twelve days after the sensitizing dose, subsequent injections of the same material produce no evidence of hypersusceptibility. Gay and Adler reported that if the euglobulins of serum are employed for sensitizing, the period of incubation may be shortened to four or five days. If during this period a second injection be given the animal is more likely to become immune than hypersusceptible. Rosenau and Anderson found that the state of hypersusceptibility increases until the twenty-first day, after which it very gradually diminishes but persists in modified form probably throughout the life of the animal.

Intoxicating Injection.—The French term, *injection déchainante*, is highly descriptive of this part of the process, as it indicates the explosive character of the manifestations that are likely to occur. This injection may be intravenous, intrameningeal, intraperitoneal or subcutaneous. The rapidity of reaction and severity of symptoms are most

marked in intravenous injection and exhibit decreasing severity in the order named. Besredka estimates that by the use of serum, approximately equivalent reactions may be produced by intravenous injections of 0.05 to 0.1 c.c., intrathecal injections of 0.066 to 0.125 c.c. and intraperitoneal injections of 5.0 to 6.0 c.c. Subcutaneous injections in experimental animals rarely produce severe or fatal reactions.

When used for the intoxicating dose the proteins are subject to the same physical and chemical agents as have been discussed in connection with the sensitizing injection. The statement of Gay and Adler that the sensitizing agent is contained in the globulin fraction of serum and the intoxicating agent in the whole serum and albumin fractions is not generally accepted and has recently been contradicted by Kato. Kato found that guinea-pigs sensitized to any of the serum fractions respond to intoxicating doses of any of the fractions but most strongly to that fraction to which they were sensitized. The aging of serum has an important influence. The toxicity of fresh serum decreases rapidly during the first ten days of preservation to about half its original power. A slight decrease occurs during the first two months, after which the deterioration is extremely gradual. Besredka has found that a serum twenty years old produced anaphylactic shock in a sensitized animal. Uhlenhuth states that he has produced anaphylactic shock with proteins from mummies.

The selection of the route of intoxicating injection depends on the character of the protein; the intravenous route is undesirable with solid proteins and even with bacteria because thrombosis and embolism confuse the picture of anaphylaxis. The minimal intoxicating dose is larger than the minimal sensitizing dose in the ratio of about 100 to 1. Wells has obtained fatal reactions with 0.000,001 gram crystallized egg-white. Fatal reactions are rarely obtained with less than 0.025 c.c. serum and, as a rule, 0.05 c.c. to 0.1 c.c. is required. We find that for laboratory demonstrations the use of 0.05 c.c. serum given subcutaneously for sensitization and 0.1 c.c. fresh serum given intravenously practically always produces fatal reactions. Of great importance is the fact that there is considerable individual variation not only in the sensitivity of the experimental animals but also in the sera employed for experiments. Wells has stated that blood serum contains so many substances that it is in reality an "extract of the animal"; hence variations such as are found in serum are not present in pure isolated proteins.

The Reaction.—The phenomena of the reaction may be discussed under three heads, the objective manifestations, the morbid anatomical changes and the functional disturbances. The reaction may be immediate or delayed, depending upon the sensitiveness of the animal, the size and mode of administration of the toxic dose and the state of deterioration of the intoxicating substance. The immediate reaction is called anaphylactic shock. In the guinea-pig the objective manifestations include rubbing of the nose, ruffling of the fur, evacuation of urine and feces, spasmodic movements of increasing severity, including

PLATE II.



Drawing of the gross appearance of the lungs of the guinea-pig in anaphylactic shock, showing marked distention and pallor. Note the overlapping of the lobes and the almost complete masking of the heart.

violent general convulsions, marked inspiratory and expiratory effort with cyanosis, exhaustion and death from respiratory failure with the heart still beating. In the dog the respiratory and convulsive phenomena are not so marked; there is violent precordial activity, marked fall in blood-pressure, diarrhea and vomiting. In man the phenomenon may show predominance of the respiratory and convulsive symptoms or of the cardio-vascular and the gastro-intestinal symptoms. Other animals show variations of the general picture outlined. The necropsy on a guinea-pig shows large, pale, distended lungs filling the thoracic cavity, cardiac dilatation, particularly of the right side, passive congestion of the abdominal viscera sometimes associated with minute hemorrhages in the gastro-intestinal tract. The lungs may show congestion, edema and small hemorrhages, but, as a rule, the distention is so marked that there is little blood in these organs. Microscopically there is marked distention of the alveoli, with rupture of their walls, constriction of the bronchioles and frequently of the small arteries. Gay and Southard describe fatty degenerative changes in capillary endothelium near small hemorrhages, as well as fatty changes in heart muscle, skeletal muscle and peripheral nerves. Beneke and Steinschneider found Zenker's degeneration particularly of the respiratory muscles, but Wells believes this to be the result of asphyxia which produces Zenker's hyalin through the increase of lactic acid in the muscle. In dogs and other animals the pulmonary distention is not marked; the important features are dilation of the heart, marked congestion and multiple hemorrhages. None of these anatomical changes is characteristic or to be distinguished from other toxic conditions. The pulmonary distention is more distinctive than any of the other changes.

From the functional point of view there have been extensive investigations of the distention of the lungs, the fall of blood-pressure, fall in temperature, delayed coagulability of the blood and alterations of the nitrogen metabolism. Auer and Lewis found that in guinea-pigs death is due to asphyxia "apparently produced by tetanic contraction of the smooth muscles of the bronchioles." This is independent of pithing, section or degeneration of the vagus, and is therefore peripheral, either in the nerve terminals or the muscle itself. Auer has shown that atropin reduces this effect, thus indicating the action upon nerve terminals. Karsner and Nutt found that there is a definite quantitative relation between the intoxicating dose of serum and the protective dose of atropin and this fact, together with the protective action of anesthetics such as ether, indicates that there may be factors involved other than mere physiological antagonisms. The exciting action on smooth muscle is not confined to the bronchiolar muscle for Schultz demonstrated a similar action *in vitro* on smooth muscle of the intestine and bladder of sensitized guinea-pigs and this has subsequently been extended to include other smooth muscle such as uterus. Pelz and Jackson have recently observed broncho-constriction in dogs during the acute shock, but although this is severe we have been unable to demonstrate acute emphysema in dogs.

The fall in blood-pressure appears in anaphylactic shock in the dog and cat but is not so highly characteristic of the reaction in the guinea-pig or rabbit. Biedl and Kraus described the condition, and it has since been studied extensively. Pearce and Eisenbrey note that it amounts to a fall of from 20 to 30 mm. mercury in the dog and believe it to be due to vaso-dilatation, particularly of the splanchnic area, due to action upon the nerve endings rather than upon the muscle. Schultz was of the opinion that the fall in pressure is due to direct action upon the heart by the toxic agent. He expressed the opinion that in the cat the fall in general pressure is due to vaso-constriction in the pulmonary circuit so that the right heart cannot empty itself. Eisenbrey and Pearce in a further study on dogs found that the functional activity of the myocardium is not primarily affected, that there is no satisfactory evidence of pulmonary vaso-constriction and that the later changes in the myocardium with the fall in general pressure result from incomplete filling of the heart consequent on the accumulation of blood in the larger venous trunks, particularly of the splanchnic area. Simonds finds that with the fall in arterial pressure, there is a fall in pressure in the superior vena cava and a rise in portal vein pressure, associated with an increase in the volume of the liver. Upon examination of the hepatic vein of the dog the vessel shows a very heavy musculature as compared with that of the herbivorous animals, and Simonds concludes that spasm of the hepatic vein and its tributaries explains the phenomena observed. Manwaring and subsequently Voegtlin and Bernheim had previously found that exclusion of the liver from the circulation prevented the appearance of anaphylactic shock, observations well in accord with Simonds' hypothesis. However, Pelz and Jackson excluded the entire abdominal circulation, and in spite of this demonstrated broncho-constriction and marked fall in blood-pressure. Thus, although numerous factors may play a part, the only fact that we can bring forward as generally accepted is that the fall in arterial pressure is associated with peripheral vaso-dilatation. Davis and Petersen observed an increase in the volume of lymph for a short time immediately following injection and again for a longer period beginning about one hour after injection. The anti ferment increases in the lymph without any change in the blood serum.

There can be no doubt that the gaseous interchange in the convulsive phases of anaphylactic shock is increased. Varying reports, however, have appeared as to the influence of anaphylactic shock upon nitrogen metabolism. Major found an inconstant decrease of nitrogen output in rabbits during shock, but this increased in the animals that survived the immediate shock to such a degree as to exceed the intake. Zunz and György found a definite increase in amino-acids, which Jobling, Petersen and Eggstein confirmed, with the additional information that the total non-coagulable nitrogen is increased. Hisanobu found a marked increase of urea nitrogen, as well as of the non-urea and amino-acid nitrogen. He concludes, as would also be apparent from Major's work, that there is an abnormally rapid destruction of

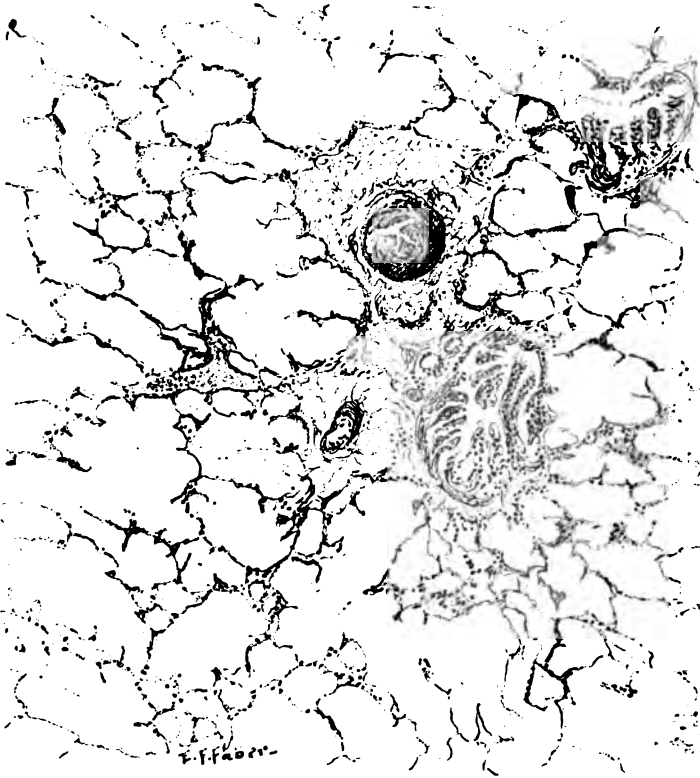


FIG. 17.—Drawing of the microscopical appearance of the lung of the guinea-pig in anaphylactic shock, showing the alveolar emphysema, constriction of the bronchioles and of an arteriole.

tissue proteins. Jobling, Petersen and Eggstein found an increase in non-specific protease with a decrease of antiferment and an associated decrease of serum proteoses; this is followed by a progressive increase in non-coagulable nitrogen, proteoses and serum lipase. They, therefore, conclude that "the acute intoxication is brought about by the cleavage of serum proteins (and proteoses) through the peptone stage by a non-specific protease." Modern opinion thus favors an increase in nitrogenous metabolism in anaphylactic shock and this may well be due to a liberation or mobilization of proteases; that the action of the latter is limited to the blood appears to us not to be conclusively proven. In spite of the increase in metabolism there is a fall in body temperature; therefore, there must be an increase in heat radiation. In lower animals the respiratory function is of great importance in heat radiation, and we suggest that the marked increase of respiration in anaphylactic shock has some bearing on this problem, but we by no means wish to exclude other factors that may play a part in the phenomenon.

The decrease in coagulability of the blood was first observed by Biedl and Kraus and since has been amply confirmed. They believed the change to be due to either a decrease of thromboplastin or an increase in antithrombin. The salts of the blood apparently are unchanged. Achard and Aynaud, as well as Lee and Vincent, found a decrease in the number of platelets, but this was not found by Biedl and Kraus. Shattuck found a delay in action of prothrombin. Pepper and Krumbhaar reached the same conclusion as Biedl and Kraus concerning a decrease of thromboplastin or an increase of antithrombin. Bulger concludes, in terms which summarize our knowledge at the present time, that the decrease in coagulability is "due to changes in that stage of the coagulation process at which thrombin is formed through the interaction of prothrombin, calcium, thromboplastin and antithrombin (?). These changes are probably due to variations in thromboplastin."

Desensitization or Anti-anaphylaxis.—If an animal recovers from anaphylactic shock its earlier hypersusceptibility is replaced by a period of resistance during which injections of the specific protein produce no demonstrable reaction. This refractory period lasts for a varying period of time up to several weeks, and although the animal subsequently becomes hypersusceptible, it rarely reaches the same degree of hypersusceptibility which it primarily exhibited. These facts were pointed out in the original investigations of Rosenau and Anderson and of Otto. Besredka has studied the matter extensively and has found that very small doses of the protein may desensitize, doses in themselves too small to lead to any observable symptoms. By repeated injections it is possible to produce such a degree of resistance that the animal may withstand doses 1000 times as great as that which proves fatal if desensitization has not occurred. The rapidity with which desensitization appears depends upon the route of injection. After subcutaneous injection it may not appear for twenty-four hours; intra-

peritoneal injections may require three or four hours for results, whereas intravenous injections may be effective in a few minutes. An experiment quoted from Besredka illustrates the rapidity and extent of the process. Guinea-pigs sensitized with egg-white exhibited fatal reactions with a toxic dose of 0.002 c.c. egg-white. An animal of this group was given 0.0005 c.c. egg-white intravenously without reaction. It did not react to 0.005 c.c., the fatal dose, given two minutes after the first injection nor to doses of 0.02 c.c., or 0.2 c.c., given at ten-minute intervals. Ten minutes later it was given 2.0 c.c. and, although visibly uncomfortable for a time, recovered. Desensitization may also be practised by four or five repeated subcutaneous or intraperitoneal injections at intervals of about two hours, the subcutaneous route requiring a longer time to be effective than the intraperitoneal route. We have found relatively large, but still sub-lethal, single doses to be most effective by intravenous injections, but less so by intraperitoneal and least by subcutaneous injection. Besredka also reports desensitization by introducing the protein into the gastro-intestinal tract but as yet this has not received widespread confirmation. Desensitization may be effective at any period during the hypersusceptible state. If a second dose be given before hypersusceptibility appears, the condition may, by subsequent injections, become one of increased resistance or immunity.

Other methods of preventing shock include the use of atropin as suggested by Auer and Lewis, of adrenalin, of chloral hydrate, administration of ether, alcohol, atoxyl and numerous other drugs. Pelz and Jackson found adrenalin most satisfactory in dogs. Karsner and Nutt found that atropin sulphate is satisfactory in guinea-pigs provided the toxic dose of serum is not too large. The use of adrenalin in guinea-pigs is unsatisfactory because of the pulmonary hemorrhage and edema which it produces. Drugs which depress the excitability of the smooth muscle of the bronchioles, those which depress nerve activity generally as the anesthetics, and those which tend to maintain blood circulation, are pharmacologically adapted to the prevention of anaphylactic shock. They do not operate as effectively after the toxic dose of protein has been given, as they do when given in time to produce physiologic effects before the onset of shock. Thomson found that exposure to the X-ray inhibits anaphylactic shock. Friedberger and Mita have suggested that anaphylactic shock may be inhibited by very slow administration of the protein. Lewis has investigated this problem experimentally and by the use of the Woodyat pump has found that "acute anaphylactic shock can be prevented in sensitized experimental animals by giving otherwise fatal doses of diluted antigen intravenously at very slow rates."

Passive Anaphylaxis.—As was shown in 1907 by the independent investigations of Nicolle, Richet, Otto and Friedemann, the serum of a hypersusceptible animal, when injected into a normal animal, will render the latter also hypersusceptible. This condition is transferred in the serum rather than in corpuscles or tissue cells. Passive ana-

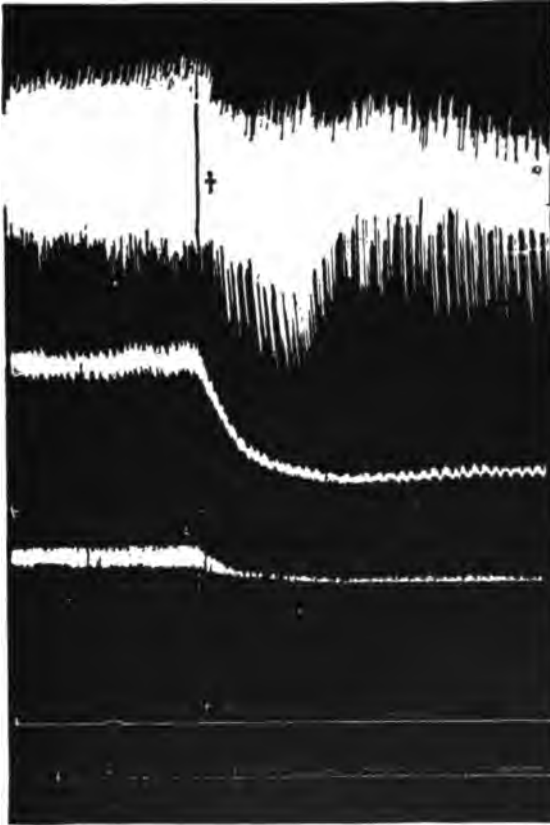


FIG. 18.—Tracing from the dog in anaphylactic shock. From above downward the tracings are: myocardiograph, blood-pressure, base line, membrane manometer, base line, signal, time in seconds. The down strokes of the myocardiograph tracing represent cardiac contractions. The perpendicular line was drawn arbitrarily through the blood-pressure curve at the point where the fall began. Corresponding points in the myocardiograph and Hurtle manometer tracings were measured and are indicated by the cross where the recording levers were not in accurate alignment. (From Eisenbrey and Pearce. A study of the action of the heart in anaphylactic shock in the dog, *Journal of Pharmacology and Experimental Therapeutics*, 4, 21, 1912.)

phylaxis may be demonstrated about four hours after intravenous administration of the serum from the hypersusceptible animal, about twenty-four hours after intraperitoneal injection and from twenty-four to forty-eight hours after subcutaneous administration. It remains at its height for about three or four days, gradually disappears in a few weeks and never exhibits the permanence of active anaphylaxis. Further study by numerous investigators has shown that passive anaphylaxis arises as the result of injection of serum from an animal in the hypersusceptible state or from an animal in the "incubation" period before sensitization can actually be demonstrated; it may also follow the injection of the serum of an animal in the anti-anaphylactic state and may be produced by the injection of an immune precipitating serum. In the last-named instance an animal is immunized to the particular protein for which passive anaphylaxis is to be produced. Doerr and Moldovan pointed out this fact, and it has been repeatedly confirmed. Scott demonstrated that the intensity of the anaphylactic shock parallels the titer of the precipitating serum. The young of sensitized female guinea-pigs are sensitive, as has long been known. The recent work of Reinald confirms this fact, but does not definitely settle the question as to whether the sensitization of the young is active or passive.

Specificity of Anaphylaxis.—That the process is specific was pointed out by the earliest investigators. It is undoubtedly one of the most specific of the biological reactions, as is emphasized by its extreme delicacy in regard to sensitizing dose. Nevertheless, group reactions appear as in the reactions of immunity. For example, a guinea-pig sensitive to sheep serum will react somewhat less violently to goat serum. Wells and Osborne have shown that cross reactions occur between gliadin from wheat and rye, and hordein from barley. The reactions, however, are strongest with the homologous protein. Nevertheless, Wells was able to separate ovovitellin and crystallized egg-white by the anaphylaxis reaction and is of the opinion that where group reactions occur the reactions are the result of common groups in the protein molecules even though the proteins may appear to be chemically distinct. If guinea-pigs are sensitized to several proteins simultaneously they will react to any of the proteins employed, but after an animal has reacted to one of the proteins, subsequent reactions to the others are less severe. Investigations conducted in this laboratory with serum proteins indicate that although desensitization is best produced by homologous sera it may be effected by biologically-related sera and by non-related sera. For such purposes considerably larger doses of the heterologous sera are necessary than of the homologous serum. Against the assumption that desensitization indicates the specificity of the reaction is the fact claimed by Banzhaf and Steinhardt that lecithin protects against anaphylactic shock. Rosenau and Anderson failed to confirm the work of Banzhaf and Steinhardt, but it may well be that a colloidal disturbance of some sort may prevent the appearance of shock and that

some similar disturbance may appear as the result of injection of heterologous sera.

In view of the great specificity of anaphylaxis it was hoped that by this means organ specificity might be demonstrated. Ranzi used extracts of liver, kidney, spleen and ovary and found that these gave species reactions with serum, but that there was no evidence of organ specificity. Pfeiffer pointed out that the organs employed by Ranzi contained blood and therefore could not be expected to show more than species reactions. He washed the organs apparently free from blood and found that animals sensitized to a given organ extract respond somewhat more markedly to that extract than to extracts of other organs, *i.e.*, there is a relative specificity. This was found to be true in somewhat lesser degree by Pearce, Karsner and Eisenbrey. Minet and Bruyant desensitized with serum and then attempted to produce shock by the organ extracts; they failed to demonstrate organ specificity. Bell has pointed out the fact that the most careful perfusion of organs fails to remove the blood completely, and it appears that Minet and Bruyant's conclusions must hold for the present. Extracts of spermatozoa and of ovary fail to exhibit organ specificity, but crystalline lens behaves as it does in the reactions of precipitation and cytolysis. Numerous investigators have shown that the lenses of different species react with each other but that serum fails to interact as either sensitizer or intoxicating body with the serum of the species from which the lens was taken.

There is no doubt that anaphylaxis produced by bacterial emulsions or extracts is specific, but reports vary as to the presence of group reactions. Delanoë holds that group reactions appear, whereas Kraus and his collaborators maintain the absolute specificity of bacterial anaphylaxis.

Theories of the Reaction of Anaphylaxis.—In order to avoid any more confusion than is necessary it seems well to review these theories in groups rather than in historical sequence. The most important difference of opinion is as to whether or not a poisonous substance is produced in the reaction. If not it would appear to be necessary to suppose that some sort of reaction occurs in the cells of the body or in the body fluids, perhaps in the nature of a liberation of energy on the part of the cells or in some form of disturbed colloidal or enzymatic balance of the fluids. If a toxic substance is formed it may be produced in the cells or in the circulating fluids. This may be the result of partial destruction of the proteins of the body or of the introduced protein, or it may appear as a new body which is formed by substances produced by the first injection coming in contact with the antigen upon second injection. This summary gives the essentials of the controversy, and a further elaboration follows.

Anaphylactic Poisons.—Richet formulated the hypothesis that the primary injection of protein produces a substance in the body which he named *toxogenine*. Upon second injection the antigen is supposed to combine with the toxogenine which has been produced during the period

of incubation and forms a toxic substance named *apotoxine*. He compares the reaction to the combination of amygdaline and emulsine to produce hydrocyanic acid. This hypothesis resembles somewhat that of Friedberger, which has been investigated intensively by many workers. Friedberger prepared a toxic substance, which he named *anaphylatoxin*, by mixing antigen, precipitating serum and complement. He obtained a precipitate by mixing sheep serum with a specific immune precipitating serum from the rabbit. This precipitate was washed, suspended in fresh guinea-pig serum for twelve hours, then centrifuged. The supernatant fluid was found to be extremely toxic for guinea-pigs. The reduction of complement in anaphylaxis has been emphasized by Friedberger in the development of his hypothesis concerning anaphylatoxin. Thomson, however, has found that this reduction is not constant and that it is in proportion to the quantity of the free antibodies in the circulation. It is insignificant when the animal has been sensitized with a small single dose of antigen, but if the animal has been sensitized by repeated doses and the precipitin content of the blood is high, the diminution in complement is likely to be marked. The symptoms following injection of anaphylatoxin include the usual clinical manifestations, with fall of temperature, retardation of coagulation of the blood and leucopenia. The poison resists heat at 56° C. for one-half hour, resists desiccation and is precipitated by alcohol. Subsequently it was found that bacteria and their antisera could be employed in the same fashion as the precipitinogen and precipitin. Doerr and Russ found that precipitates are toxic without the addition of complement, and in view of this fact and the production of passive anaphylaxis by precipitating sera, reached the conclusion that precipitin and the substance produced by the primary injection in anaphylaxis are inseparable. Kraus and his co-workers have contradicted this parallelism and point out that the guinea-pig is a poor producer of precipitin; rabbits may produce a powerful anaphylactic substance without producing precipitins; goats produce precipitin readily but have a serum incapable of conferring passive anaphylaxis. Biedl and Kraus pointed out the fact that injections of pepton produce symptoms similar to anaphylaxis in the dog. Karsner has confirmed this in the guinea-pig. Biedl and Kraus found that following injection of pepton into a dog the animal subsequently fails to react to anaphylaxis and hence they formulated the hypothesis that the poison of anaphylaxis is a pepton-like body. Doerr offered the hypothesis that the actual disturbance is in the physical character of the blood. He assumes, however, that this disturbance is produced by a toxic agent originating in complement. The complement is supposed to contain the toxic substance held in check by an antagonistic substance; the latter is adsorbed by precipitates or bacteria, thus liberating the toxic substance. The further investigation of the so-called anaphylatoxin led to the discovery by Keysser and Wassermann that a similar substance could be produced by the action of complement on barium sulphate or kaolin. Besredka then found that placing fresh serum upon pepton agar produces a toxic fluid which induces

symptoms identical with those from anaphylatoxin. Bordet found that the action of fresh serum upon agar in solution produces a similar toxic substance. Novy and De Kruif have published very extensive studies upon toxic materials in a measure similar to the anaphylatoxin. It is found that the action of serum upon agar intensifies the toxic power of the agar. They have shown that agar and other non-protein colloids produce anaphylactoid symptoms.

The poison, if there be such in anaphylaxis, is not dependent on the presence of any antibody or other substance within the cells of the sensitized animal, because it can be produced *in vitro*; neither is it dependent on antigen, inasmuch as barium sulphate and kaolin serve a similar purpose; nor is it dependent on complement, for, as Doerr has shown, it can be produced without the action of fresh serum. Besredka maintains that the anaphylatoxin produces no symptoms by sub-dural injection and that it kills only upon intravenous injection. Besredka has found that pepton does not interfere with true anaphylaxis in the guinea-pig, but that it does inhibit the action of anaphylatoxin. Furthermore, the state of anti-anaphylaxis which protects an animal against a massive dose of the antigenic substance and therefore prevents anaphylactic shock has no such protective influence upon anaphylatoxin. These arguments as well as those presented in the subsequent section on the cellular theories of anaphylaxis serve to show that there is probably no poison, which can be produced *in vitro*, that leads to the development of a condition identical with true anaphylaxis. Certain features of this discussion will be referred to under the heading of Anaphylactoid Phenomena.

Cellular Theories.—The conflicting views are that either a poison is produced within cells, or that some disturbance of cells appears independently of the production of a poisonous substance. Gay and Southard, influenced perhaps by the prevailing conceptions of immune reactions and impressed by the cellular degenerations seen in their animals, emphasized the intracellular character of the reaction. They assumed that the injected protein contains a substance, anaphylactin, which is eliminated from the body extremely slowly, in contrast to the fairly rapid elimination of the other constituents of the protein. "The anaphylactin, however, remains and acts as a constant irritant to the body cells, so that their avidity for the other assimilable elements of the horse serum (or protein), which have accompanied the anaphylactin, becomes enormously increased. At the end of two weeks of constant stimulation on the part of the anaphylactin, and of constantly increasing avidity on the part of the somatic cells, a condition has arrived when the cells, if suddenly presented with a large amount of horse serum, are overwhelmed in the exercise of their increased assimilating functions and functional equilibrium is so disturbed that local or general death may occur." This theory was supported by their statement that the sensitizing fraction of serum is contained in the globulin fraction and that the other elements of serum may serve to produce shock. They could not produce a toxic body by mixing the

serum of sensitized guinea-pigs and horse serum. The fact that further investigation, as for example that of Wells and of Kato, has failed to demonstrate a manifest difference between sensitizing and intoxicating fractions of the protein, is an argument against this hypothesis. Friedberger's original conception was that the primary injection leads to the development of receptors in the cells but in such small amounts as not to be liberated into the blood stream. These "sessile" receptors are responsible for an increased affinity of the cells for the antigen, the consequent disturbances resulting from the rapid anchoring of the protein by the cells. If injections are repeated before the anaphylactic state is developed the receptors are formed in large amounts and appear in the blood stream as precipitins. This hypothesis accords well with the modern conception of immunity and anaphylaxis save for the assumption that the sensitizing substance and precipitins are identical. This theory was followed by Friedberger's anaphylatoxin theory. Somewhat more concrete is the hypothesis of Vaughan and Wheeler. After a long period of study of toxic fractions of bacterial and other proteins by Vaughan and his co-workers, the following statement in regard to anaphylaxis was made. "When a foreign protein is introduced into the blood or tissues it stimulates certain body cells to elaborate the specific ferment which will digest that specific protein. When this protein first comes in contact with the body cells, the latter are unprepared to digest the former, but this function is gradually acquired. The protein contained in the first injection is slowly digested, and no ill effects are observable. When subsequent injections of the same protein are made, the cells prepared by the first injection pour out the specific ferment more promptly, and the results are determined by the rapidity with which digestion takes place. The poisonous group in the molecule may be set free rapidly, and in amounts sufficient to produce symptoms, or to kill the animal." Jobling and his co-workers, however, have reached the conclusion that the development of proteases in the blood is not dependent upon antibodies and is not specific. Vaughan replies to this objection that "we have only transferred the problem of specificity from the development of a specific enzyme to the specific uncovering of a non-specific enzyme." Undoubtedly, the bodies studied by Vaughan are extremely toxic. As an example, he found that the product of 1 gram of casein is sufficient to kill 800 guinea-pigs. We are not ready to admit that toxic substances of this sort produce clinical and pathological changes that are identical with anaphylaxis. Weil has given the participation of the cells most extensive study. He considered that the cells are of the utmost importance in the destruction and elimination of foreign protein and that in the course of this process they construct an antibody. The union of antigen and antibody within the cells gives rise to the serious disturbances which constitute anaphylaxis. His excellent work was interrupted by his death in the service of his country, but his hypothesis is one which serves equally well in the phenomenon of desensitization and in anaphylaxis. In support of the assumption that the primary

change is in the cells may be considered the work of Schultz, Dale, Woods and others with isolated sensitized organs containing smooth muscle. These organs, washed free of blood, responded specifically to the protein with which the animal was sensitized. Of considerable value was the experiment of Pearce and Eisenbrey, who transfused dogs so that the blood of a sensitized dog circulated in the body of a normal dog and vice versa. Under these circumstances the intoxicating dose of the antigenic protein produced symptoms in the sensitized dog with normal blood, but no symptoms in the normal dog provided with blood from its sensitized fellow. Coca confirmed this with the guinea-pig. Although Manwaring and collaborators have found that perfusion of rabbit heart indicates that anaphylactic shock is entirely humoral, subsequent work of Manwaring and Kusama with perfusion of guinea-pig lungs showed that the cells of the lungs of sensitized animals respond by bronchiolar constriction to perfusion with antigenic serum. They also found that perfusion of normal lungs with a mixture of the blood of a sensitive animal and antigen also produces bronchiolar constriction. None of the experiments so far outlined establishes definitely the parts the cells play, for, as Bell points out, none of these methods has completely removed the native blood from the organs. We know that minute amounts of certain protein poisons are highly toxic, and it may be that the amount of blood left in a perfused organ is sufficient for the production of a humoral poison. Nevertheless, studies of passive anaphylaxis tend to confirm the conception of cellular participation. Weil has pointed out that simultaneous injection of a serum, capable of producing passive anaphylaxis, and its antigen fails to produce symptoms. A certain interval of time must elapse before an animal becomes passively anaphylactic, an interval in which it is presumed the cells either anchor or develop the sensitizing substance. Isolated organs fail to respond to the antigen until a certain time has elapsed. The time element depends to a certain extent upon the mode of injection, but is never less than several hours even with intravenous injection. This fact, in association with the experiments in active anaphylaxis, *in vitro*, with perfusion and with isolated organs, all tend to support the conception that the participation of the cells is of fundamental importance in the reaction. Weil has studied further the phenomenon of desensitization and finds that the reaction between the cellular antibody and the antigen follows in a general way the Danysz phenomenon (see page 50). By the fractional injection of antigen the substance in the cells takes up the antigen so that subsequent additions of antigen produce little effect. He states that "partially combined cellular antibody manifests a marked diminution in its affinity for fresh antigen." Thus the conception of cellular participation fits the demonstrated facts of passive anaphylaxis.

Physical Theories.—These have been less susceptible to experimental proof than other theories because of the limitations of technic. As has been mentioned, Doerr conceived the idea that adsorption of the supposed antagonistic substance of complement by bacteria or precipi-

tates liberates the toxic substance of complement. This theory omits consideration of the cellular participation and needs further elaboration to be acceptable. Of more significance is the fact demonstrated by Jobling, Petersen and Eggstein that anaphylactic shock "is accompanied by (a) the instantaneous mobilization of a large amount of non-specific protease, (b) a decrease of antiferment, (c) an increase in non-coagulable nitrogen of the serum, (d) an increase in amino-acids, (e) a primary decrease in serum proteoses." They conclude that the "intoxication is brought about by the cleavage of serum proteins (and proteoses) through the pepton stage by a non-specific protease" and that "the specific elements lie in the rapid mobilization of this ferment and the colloidal serum changes which bring about the change in antiferment titer." From our discussion of the cellular participation in anaphylaxis the conception of Jobling cannot be accepted as entirely satisfactory, but it has more ground in demonstrated fact than any of the other physical theories. Support for Jobling's conception is furnished by Bronfenbrenner and others. Bronfenbrenner finds, however, that the state of dispersion of the colloids is important in maintaining the ferment-antiferment balance and that simply bubbling ether through serum decreases the antitryptic activity probably because of an increased dispersion of colloidal particles. A similar decrease of antitryptic activity of the blood follows a mixture of antibodies and antigen. This theory may be applied to desensitization by assuming that the small intoxicating dose inhibits antiferment, that the proteases then operate and that the split products act as antitrypsin, thus preventing the toxic effects of subsequent injections. Danysz hypothesizes that anaphylaxis is an intracellular or intravascular disturbance of digestion or a combination of the two. The disturbance of digestion consists in an inability of the organism rapidly to transform the colloid antigen into crystalloids. The symptoms are produced by a sudden alteration of equilibrium between the sol. and gel. state of the colloids which enter into the composition of the cells and of the blood. His conclusion that acute anaphylaxis is due to intravascular changes in the animal is in contradiction to what we believe to be well demonstrated facts. Kritschewsky found that the sap of a certain plant, *cotyledon scheideckeri*, precipitates blood proteins, agglutinates and hemolyzes erythrocytes. Symptoms in animals following injections into the circulation or subcutaneously resemble anaphylaxis and are due, Kritschewsky believes, to a change in degree of disperseness of the plasma colloids, and he therefore assumes that anaphylaxis is of the same nature. We do not concede that Kritschewsky worked with true anaphylactic shock, as the pathological findings in his animals lack the uniformity of those seen in true anaphylaxis. Similarly we object to the experiments of Doerr and Moldovan who produced toxic symptoms by the injection of water colloidal solutions of silicic acid, also of nucleinic acid and of dialyzed iron hydroxid. Kopaczewski found that the injection of serum rendered toxic by addition of bacterial suspension or colloidal gels., when injected into animals reduces the surface tension of their

blood three or four dynes, from which reduction the animal gradually recovers. Upon investigation of the electrical potential of sera it was found that a current of eight volts shows a precipitate at both electrodes in the case of normal serum, but that with a so-called anaphylactic serum the precipitate collects almost entirely in the negative pole. Although Besredka's former idea that the injected serum contains a separate sensibilisinogen which leads to the formation of sensibilisin in the cells, and an antisensibilisin which combines with sensibilisin upon the second injection of the serum is not in accord with prevailing ideas, yet he was one of the first to propose a physical theory. Thus he stated in a general way the majority of the facts seem to indicate that the phenomena of anaphylaxis and anti-anaphylaxis are reduced to the action of precipitation and adsorption which upset the mutual relations of the colloids. Besredka no longer insists upon the separation of the two elements of protein, but is of the opinion that the important site of reaction is in the nerve cells. He believes that the second injection of the protein meets with the preformed sensibilisin in the cells and produces there either a liberation or absorption of energy, thermal or otherwise, and that this reaction leads to the phenomena of anaphylactic shock. He compares the reaction to the mixing of water and sulphuric acid. If the water is added suddenly to the acid there is an explosive liberation of the heat of hydration. If the water is added slowly, the heat is generated more gradually and no serious manifestations take place. So with anaphylaxis, if the injection is in a single large dose, anaphylactic shock is produced, but if several small doses are given, there is a series of very slight shocks leading to no serious disturbance and so desensitizing the body that serious results cannot follow a subsequent large injection. Besredka argues that the inhibitory effect of anesthetics supports his contention that the nerve cells are of great importance in production of anaphylactic shock, but the work of numerous investigators shows that the broncho-constriction and fall in blood-pressure occur in spite of anesthesia, and that the reaction may be fatal if the intoxicating dose be sufficiently large. Bronfenbrenner points out that anesthetics increase the antitryptic power of the blood 100 per cent. or more, thus inhibiting the liberation of proteases and the consequent production of toxic split products. As a further objection to Besredka's conception is the fact that the experiments with isolated organs and perfusion demonstrate that smooth muscle reacts and that the phenomenon is by no means confined to the nerve cells. When calorimetric and metabolism experiments can be performed with nerve tissues, definite information can be obtained in regard to energy changes in these tissues in anaphylaxis.

Anaphylactoid Phenomena.—In the discussion of the theories of anaphylaxis references have been made to anaphylatoxin and certain similar substances. As has been pointed out, these substances may be protein in character, may represent certain decomposition products of protein, or may be non-protein colloids. It is even maintained that arsphenamine is to be included in this category of colloids. The in-

vestigation of these substances has had an important bearing on the development of theories of anaphylaxis, because if these can be compared to the supposed toxic substance of anaphylaxis it would seem reasonable to suppose that anaphylaxis has as its basis a colloidal disturbance. Many of those who have worked with these substances have not been strict in their use of the term anaphylaxis and have depended in large part on the clinical manifestations following the injections of these agents. From time to time certain investigators have indicated that more intimate study would prove that anaphylaxis and the phenomena following the injection of these colloids are not identical. Manwaring and Crowe, for example, found that occasionally there appears in anaphylaxis occlusion of pulmonary blood-vessels by thrombi and used the term pseudo-anaphylaxis. The problem has recently been investigated extensively by Hanzlik and Karsner. The experiments in this series of studies were controlled by gross and microscopic studies of the viscera of the animals after death. More than thirty colloidal agents were studied by a variety of methods, including intravenous injection, studies of perfused organs, protection by atropin and epinephrin, as well as test-tube studies of the action of the agents upon blood-corpuscles. Many of the agents studied produce serious disturbances of circulation and others produce equally serious disturbances of respiration. In the case of none of these colloids was it possible to demonstrate that the clinical and morbid anatomical phenomena, taken collectively, are identical with those of anaphylaxis. The symptoms provoked can all be explained on grounds other than the assumption that we are dealing with anaphylaxis. Even in the case of agar, where bronchial constriction and pulmonary distention are well marked, the common occurrence of thrombi both in the living animal and in perfused lungs definitely excludes an identity with anaphylaxis. These phenomena may, therefore, be considered as of colloidal nature and may well be referred to as "colloid shock." Pepton produces symptoms and signs more nearly like those of true anaphylaxis than the other substances studied, but the fact that pepton more frequently produces thrombosis, hemorrhage and edema of the lungs than is the case in true anaphylaxis, would place pepton poisoning in the group of anaphylactoid rather than anaphylactic phenomena. Similarly the injection of primarily toxic sera such as ox serum and eel serum into the guinea-pig produces certain circulatory disturbance with hemorrhage and edema. It seems probable that the toxicity of some of the substances of protein nature or the decomposition products of protein may depend for their activity upon the presence of histamine. The poisonous character of histamine depends in no way upon previous sensitization, is primarily toxic and therefore may be included as anaphylactoid in its action.

Summary.—With a strict adherence to the conception that anaphylaxis constitutes that state of hypersusceptibility to a given substance, which has been induced by a previous injection of the same substance we may conclude that the mode of the second injection determines the

particular manifestations observed. The sensitizing fraction of the protein, if there be any such fraction, has not been isolated nor has the intoxicating substance. If the second dose be given in mass, anaphylactic shock results. If, on the other hand, divided small doses are given the state of the organism is so changed that severe anaphylactic shock does not appear. In agreement with Besredka, we believe that desensitization produces a series of minor shocks but believe that the explanation lies rather in the work of Weil than in the hypothesis of Besredka. In other words, there is a partial saturation of the sensitizing substance within the cells, so that any subsequent union cannot produce the intensity of reaction that would have been produced by a massive injection. The time that must elapse for the production of passive anaphylaxis, as well as the other experiments offered in evidence, support the conception that some change must occur in the cells in order to produce sensitization. The nature of the combination between the specific protein and the substance within the cells or the influence of the protein upon the cells is not definitely known, but the data offered in review appear to rule out the probability that definite toxic bodies are formed. Similarly the nature of the primary changes in the cells upon second injection cannot be identified; as to whether there is a liberation of energy of some sort or a disturbance of colloidal relations must still be the subject of investigation. The specificity of the reaction is similar to that of other biological reactions and is subject to similar limitations of the group phenomenon. Nevertheless, we find in anaphylaxis a most specific phenomenon, which is approached in delicacy only by the reactions of precipitation and of complement fixation.

The Relation of Anaphylaxis to Immunity.—If desensitization of an anaphylactic animal is carried on for only a short time the period of desensitization is relatively brief, but, on the other hand, if the vaccination be continued the animal may be rendered resistant. This indicates a close relationship between the two phenomena. We do not propose to discuss this at length because of the intricacy of the subject. Weil pointed out in his earlier experiments by saturation of the animal with proteins that although the animal may become immune in so far as his body fluids are concerned he may remain hypersensitized in so far as his cells are concerned. Manwaring and Kusama found that the lungs of guinea-pigs immunized to a certain protein, when washed free of blood, were still sensitive to perfusion with the protein in question. We, therefore, revert to the conception of Weil that immunity is in large part exhibited in protective power of the blood and body fluids. In the state of anaphylaxis this immunity has not been established in the fluids and therefore the cells can be directly operated upon by the antigen. If, on the other hand, the animal is immune his blood and fluids combine with the antigen so as to protect the cells. The direct bearing of this upon diseases in man is a matter of speculation. It seems possible, however, that during the period of incubation of an infectious disease the animal, as suggested by Danysz, likewise

passes through the period of sensitization to the infecting organism. When, therefore, the infecting organism or its products are present in sufficient amounts the manifestations of disease appear in the form of what may be termed an acute or sub-acute anaphylaxis. As time goes on this process is transformed into an immunity and the disease undergoes cure. In this latter state it may be assumed that the body fluids have developed a sufficient amount of protective substance so that the cells are no longer susceptible to attack. This would satisfactorily explain the self-limitation of infectious disease. By assuming that injury of the cells may have become so serious as completely to interfere with life processes, death may ensue, or if the disturbance is not so severe the condition may exhibit the chronic complications which so frequently follow acute infection.

CHAPTER XI

HYPERSUSCEPTIBILITY IN MAN

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SERUM DISEASE.

THE DELAYED REACTION.

THE ACCELERATED REACTION.

ANAPHYLACTIC SHOCK IN MAN.

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CUTANEOUS REACTIONS TO VACCINE VIRUS.

CUTANEOUS REACTIONS IN GLANDERS.

OTHER CUTANEOUS REACTIONS.

Introduction.—The manifestations of hypersusceptibility in man can be classified into two groups, those in which a definite previous sensitization has been effected and those in which no such sensitization is known or can be conclusively proven. In the former group are included a relatively few cases of anaphylactic shock and the widely-observed phenomenon called serum disease. In the latter group are those individuals who are abnormally sensitive to a wide variety of substances. These may gain access to the body from the air, through the respiratory tract, skin or conjunctiva or through ingestion of foods which contain the specific substance. In addition to air contacts, direct contacts with plants and animals, which may or may not serve to produce dusts, may also lead to dermal manifestations of hypersensitiveness.

Serum Disease.—*The Delayed Reaction.*—The serum treatment of various diseases has given ample opportunity for the study of the symptom complex called by von Pirquet and Schick serum disease. This follows with extreme frequency upon subcutaneous, intravenous or intrathecal injections of animal sera employed for therapeutic purposes and may be delayed or accelerated. The symptoms may develop after a primary or series of primary injections and constitute the delayed reaction. These symptoms appear from six to twelve days after the

injection, and in our experience have been most frequent after ten to eleven days. The most noticeable and most common symptom is a skin eruption which is usually urticarial but may be a patchy or diffuse erythema, a scarlatiniform or a multiform eruption. Edema may appear in the lips, eyelids, face or other parts of the body and rarely may effect the larynx. According to Longcope, "in one instance a transient hemiplegia was supposed to be caused by local edema of the meninges." We have seen one case in which a broncho-pneumonia, following a prophylactic injection of serum appeared to be the sequence of an edema of the bronchi. There is often lymph-node enlargement, which may precede the eruption and may be accompanied by enlargement of the spleen. There is likely to be a moderate fever, headache, malaise and occasionally nausea and vomiting. Multiple joint pains, increased by motion, but without tenderness, redness or swelling, are common in severe cases. Albuminuria appears in 5 to 9 per cent. of the cases, and Longcope has found that there is likely to be salt and water retention with little or no disturbance of nitrogenous elimination. There may be a primary leucocytosis, followed by a leucopenia, which latter shows an absolute increase of lymphocytes. The condition usually lasts twenty-four, forty-eight or seventy-two hours and occasionally is prolonged to twenty days or more. Relapses may occur, more particularly after the use of large amounts of serum.

Several factors enter into the occurrence, severity and duration of the disease, the larger doses giving more frequent occurrence, greater severity and longer duration. There are certainly individual differences in the resistance of patients and probably individual differences in specimens of serum. Sera from different species exhibit differences in toxicity for man, that of the ox, according to Kraus, being less likely to produce serum disease than that of the horse. The globulin precipitation or so-called concentration of horse serum in the preparation of antitoxins reduces the toxic manifestations in man.

The Accelerated Reaction.—Frequently repeated injections of serum at properly spaced intervals may lead to a state of resistance or immunity, but this is practically never permanent. Following a primary injection or series of injections, there usually develops a state of hypersusceptibility. This condition does not precede the appearance of the delayed reaction and does not precede the tenth day after injection, even if the delayed reaction fails to appear. Repeated doses of serum at short intervals delay the appearance of hypersusceptibility. The height of sensitiveness is reached in from two to three months, after which it slowly subsides but probably never entirely disappears. We have observed accelerated reactions nine and fourteen years after primary injection. The hypersusceptibility exhibits itself only on injection of the protein and is specific for the species from which it originated. Following the second injection of the protein or serum there is occasionally no acceleration of reaction, but if accelerated it may be moderately or markedly so, the last producing the so-called immediate reactions. These immediate reactions may be local, appear about the site

of injection in from a few minutes to an hour or two and show edema, erythema or urticaria. There may also be a general immediate reaction which appears in from twelve to twenty-four hours and in addition to the usual symptoms and signs of serum disease may be accompanied by severe asthmatic form of dyspnea, cardio-vascular disturbances with cyanosis, collapse, chills, nausea and vomiting and renal disturbances including complete suppression for several hours. If the second injection is given when hypersusceptibility is not marked, as for example after a small primary dose, or several years after a primary dose, the accelerated reaction is not likely to be immediate but appears in from two or three to five or six days. Under these circumstances the reaction may appear as an ordinary case of serum disease or may be more severe.

Anaphylactic Shock in Man.—There is little doubt that the accelerated reactions of serum disease bear in some way a relation to anaphylactic shock. During the period of hypersusceptibility in man the subcutaneous administration of serum rarely if ever produces death, in spite of the fact that the clinical symptoms may be extremely severe. On the other hand, intravenous injections have been reported to produce death following symptoms closely resembling those of anaphylactic shock in animals. Reports of accidents of this sort led to the fundamental investigations of Rosenau and Anderson, which have been described. Injections of serum into the spinal canal have been followed by fatalities, but an analysis by Auer of the reported cases leads him to believe that for the most part these deaths were due to other causes than anaphylaxis. Miller and Root, in analysis of death following subcutaneous administration of horse serum, find that death in some instances was probably caused by status thymo-lymphaticus and that in other cases the cause of death had not been demonstrated to be anaphylactic. The clinical and pathological picture of fatalities has in most instances not been clearly described. Nevertheless, Boughton has recently reported a case in which a man, the subject of bronchial asthma when near horses, died upon being given intravenously one minim of normal horse serum. Autopsy showed enormous distention of the lungs with congestion of other viscera and numerous small hemorrhages. This apparently is an instance of true anaphylactic shock in man, and it cannot be doubted that such accidents occur. Caution must be exercised, however, in attributing death to anaphylaxis because of the numerous other conditions which may lead to sudden death, particularly in the course of acute infectious diseases.

Natural Hypersusceptibility.—The recent scientific investigations of hay fever and its various modifications, as well as asthma, eczema, other diseases of the skin, angio-neurotic edema and certain gastrointestinal disturbances, have shown that a considerable number of these cases are hypersusceptible to proteins of various origins. The skin reactions, to be described subsequently, and the effect of specific treatment both demonstrate the etiological influence of the special proteins. The evidence presented from large clinics devoted to the study of these conditions leaves no doubt concerning the fact that many of these cases

are instances of hypersusceptibility. The sensitive state appears to be inherent in the cells of certain individuals, and although not directly inherited, Cooke and Van der Veer have found that the tendency to spontaneous sensitization appears to be heritable, that it follows the law of Mendel and appears as a dominant character. Nevertheless, there is a possibility that sensitization may be acquired in some manner. Cooke, Flood and Coca maintain that artificial sensitization cannot be produced by pollens. Heyl, however, has obtained from the pollen of ragweed an albumin, a proteose and a globulin and found that mixtures of the albumin and proteose possess definite sensitizing properties upon animal inoculation. Individuals who have been given horse serum therapeutically become somewhat sensitive to subsequent injections of horse serum, but only in rare instances is the sensitiveness shown as a coryza or asthma when near horses. The chance of sensitization by injection of other proteins is not great. The possibility of sensitization by virtue of the material gaining access to the body through the respiratory or intestinal surfaces is apparently remote. There is little satisfactory evidence that protein materials in the form of dust gain access to the circulation through the respiratory membrane. Ulrich, however, reports the experimental sensitization of guinea-pigs by nasal insufflation of pollens and of horse serum, but reports that rabbits cannot be so sensitized. It is impossible, under these circumstances, to exclude the possibility that the material is ultimately swallowed, and sensitization effected through the intestinal tract. The ingestion of proteins as foods ordinarily leads to such changes in the protein in the process of digestion that the absorption of the products cannot produce sensitization. On the other hand, it is known that if given in large amounts and given under certain circumstances native protein may gain access to the blood stream through the intestinal tract. Rosenau and Anderson maintained that sensitization could be effected by feeding horse serum to guinea-pigs, but the failure of Besredka, as well as of other investigators, to confirm this leaves the matter in some doubt. As against the acquisition of hypersusceptibility in hay fever, Dunbar and also Cooke, Flood and Coca have found that patients may be sensitive to the pollens of plants indigenous to foreign countries and with which the patients have never come in contact.

Hay fever, rose fever and similar disturbances are due to the pollens of certain plants and the flowering period of these plants determines the seasonal prevalence of the disease. The pollens responsible are those which are disseminated by winds; those plants which are pollinated by insects do not produce hay fever. Scheppegrill points out also that the direct effects of pollens are of importance as they may be locally irritant to both normal and hypersusceptible individuals, either because of the mechanical effect of spiculated pollens or because of the discharge from the pollen of irritant juices. Local reactions may be increased by anatomical malformations in the nose and pharynx, such as deviation of the septum, polyps, adenoids, and the condition may entirely subside following correction of these abnormalities. Strouse

and Frank claim that the attacks may be intensified and prolonged because of a concurrent acute or sub-acute bacterial infection, which perhaps permits greater absorption of the pollen protein. The condition may be so severe as to be called asthma, and in addition to respiratory phenomena may show erythematous and urticarial eruptions. Similar conditions are met with in certain individuals sensitive to the effluvia of horses, rabbits and other animals. It is well known that the ingestion of certain foods, such as egg albumin, shell fish, strawberries, may give rise to serious intestinal disturbances and that these may occasionally be associated with skin eruptions or respiratory disturbance. In sensitive individuals contact of the skin with plants or animals, to the protein of which the individual may be sensitive, leads not uncommonly to cutaneous eruptions. These, however, are not likely to be very severe or of long duration. The inflammation of the skin in ivy or sumac poisoning is not to be included in this group, because the irritant agent is probably not of protein nature, but rather an acid-resin. Eczema and perhaps certain other skin diseases may also be due to hypersusceptibility, and it is found that this is exhibited rather toward food products than toward other forms of protein. Furthermore, certain of these cases of asthma, eczema, etc., may be due to bacterial proteins as well as those of higher plants and of animals.

The hypersusceptibility of the sort discussed in this section differs from induced hypersusceptibility in two important respects. In the first place, the degree of sensitiveness is extreme. This may be illustrated by the case reported by Boughton, quoted above, in which one minim of horse serum produced death. It is further illustrated by the fact that hay fever, asthma and other similar conditions are induced by what must necessarily be an extremely small amount of protein in the atmosphere. In the second place, the sensitization is not limited strictly to a single protein. Longcope classifies these individuals roughly as those "who react to the sera of animals; those who react to eggs, or the sera of fowls; those who react to the extracts of shell fish and those who react to the protein of plants." Within each group the individual may be sensitive to the protein of several species. As has been pointed out by Walker, those who react to bacteria frequently react to several varieties of organisms. Furthermore, individuals may occasionally show reactions to two or three of the large groups indicated by Longcope. Of further interest in regard to specificity is the fact that apparently within a given species, proteins of somewhat different origin may not produce identical reactions. For example, skin reactions may demonstrate sensitiveness to horse dandruff and not to horse serum. Desensitization may be produced by careful and prolonged vaccination, but as in experimental animals the desensitized state does not persist for a very extended period, it may be necessary to repeat the vaccinations every six months, every year, or at such other periods as the individual case requires. The possibility of passive sensitization in natural hypersusceptibility is illustrated by a case reported by Ramirez. A man who had never shown any hypersensitiveness to proteins

was transfused with the blood of a donor who was a victim of horse asthma. The recipient, two weeks later, while driving in a carriage, was seized with a typical asthmatic attack and subsequently showed a positive skin reaction to horse dandruff. This apparently is a case of passive transfer of a natural hypersusceptibility. No data have been collected to show whether such a variety of passive sensitization is permanent in man or exhibits the same evanescent character as occurs in animals. Passive sensitization of animals has been produced by Koessler, but Cooke, Flood and Coca, as well as Ulrich, have been unable to confirm this. Our own experience with one case of human hypersusceptibility to rabbit serum failed to demonstrate passive transfer into guinea-pigs.

Tests for Hypersusceptibility.—The manifestations of hypersusceptibility may be general or local, depending on the mode of inoculation and the amount of material employed. If the dose can be carefully regulated, the hypersusceptible state may be demonstrated by inducing a general reaction, as in the tuberculin reaction. Owing to the fact that individuals may be extremely sensitive to certain proteins, as in the case reported by Boughton (see page 230), the use of the general reactions for diagnostic purposes is limited to those in which severe general reactions are not likely to appear. The local reactions give equally satisfactory information in man and are devoid of serious results. These local reactions are based fundamentally upon the studies of Arthus, published in 1903. He found that if animals are given several subcutaneous injections of normal horse serum at three- or four-day intervals, the first three injections are absorbed readily, but the fourth is followed by a local inflammatory reaction and subsequent injections are likely to be followed by more severe inflammation, necrosis and gangrene. Animals rendered sensitive by these first two or three injections could be killed by intravenous or intraperitoneal injections. The Arthus phenomenon was early employed as a means of detecting hypersusceptibility resulting from bacterial invasion, but it has now found widespread employment in the detection not only of the presence of changes incident to infectious disease but also for the determination of sensitiveness to a large number of proteins of animal and vegetable origin. Although hypersusceptibility may exhibit itself in respiratory phenomena as in hay fever, horse asthma, etc., or in gastro-intestinal disturbance, as in sensitiveness to egg-white, definite local reactions may be evoked by the introduction of the proteins into or under the skin and these local reactions may be accompanied by general symptoms, such as fever, headache, malaise and transitory leucopenia followed by slight leucocytosis with an associated esinophilia.

Toxins in Hay Fever.—The studies of Dunbar assumed that the irritant agent in pollens is a toxin. He based this conclusion on the fact that he could prepare a so-called antitoxic serum "pollantin" by immunizing animals and subsequently claimed that he could demonstrate antibodies by precipitin and complement-fixation tests. Clowes found positive precipitation and complement fixation in some but not all cases before

the beginning of the hay-fever season, which disappeared for a few weeks after specific desensitization. On the other hand, numerous other investigators have failed to demonstrate such reactions, and this phase of the question must be considered unsettled. Dunbar claimed that treatment with the antiserum "pollantin" produced specific effects, but Weichardt maintains that equally good results are obtained with the serum of normal animals taken in the summer season. Cooke, Flood and Coca were unable to demonstrate immune reactions in the sera of rabbits inoculated repeatedly with the pollens of ragweed and of redtop. Other objections to the toxin conception include the fact that the majority of normal individuals are, practically speaking, absolutely resistant to the pollens and fail to react to doses 1000 times the dose which produces reactions in susceptible cases. This is not in accord with the finding in regard to any other of the known toxins. Apparently normal individuals may resist diphtheria toxin, but Cooke and Van der Veer have pointed out that such resistance depends upon the presence of demonstrable antitoxin, which is not true in resistance to pollens. By mixing the "pollantin" and pollens and then testing by an ophthalmic reaction in sensitive individuals Dunbar's assistant, Prausnitz, plotted a curve of neutralization, but Wolff-Eisner found that this curve does not follow the law of multiple proportions and is therefore not similar to other toxin-antitoxin combinations. There seems, therefore, little ground for assuming that the pollens contain a special toxin and the subsequent work with hay fever and similar conditions indicates that they represent a condition of hypersusceptibility to proteins or to protein decomposition products.

Technic of Cutaneous Tests.—If the antigenic proteins are already in solution, as is the case with blood serum, no especial treatment is required other than suitable dilution under strictly aseptic precautions. If the protein is in solid form, as in the case of vegetable proteins and other cellular forms, extracts are required. The studies of Walker and of Wodehouse on the preparation of materials for the tests have been of the utmost importance. These are independent of the preparation of the various tuberculins, which will be discussed subsequently. They found that an excellent dried preparation of serum could be obtained by precipitating with several volumes of acetone, washing the precipitate centrifugally twice with alcohol and with ether, and drying to a powder. The powder may be applied to an incision in the skin and dissolved with N/10 NaOH solution. Bacteria are cultivated on solid media, washed centrifugally in salt solution, then twice in absolute alcohol with 0.5 per cent. phenol added, twice in ether and then dried to a powder, which may be used as is the serum powder. Cereals, nuts and other seeds, roots and tubers, fruits, leaves and stems are extracted in water, precipitated with 95 per cent. alcohol, washed with 95 per cent. alcohol, absolute alcohol, ether and desiccated over hydrochloric acid. Hair and dandruff of animals may be employed as a dissolved extract in 14 per cent. alcohol, but for more accurate studies, dried preparations of acid metaprotein, alkali metaprotein and pepton extracted from the material are employed.

The methods of inoculation include introduction of the protein into abraded surfaces and intracutaneous injection through a fine needle. In special instances, as, for example, in the use of tuberculin, the material may be incorporated in an ointment and carefully rubbed into the skin; this is the so-called percutaneous test. Somewhat similar to the cutaneous tests is the ophthalmo-reaction, more particularly applied in tuberculin tests, where the material is instilled into the conjunctival sac. Subcutaneous injection of material is also resorted to, again with tuberculin rather than with other

substances, but the determination of results is by means of the general rather than the local reaction. As with other reactions, controls are a necessary part of these tests. The cutaneous test, by which is meant introduction of material into an abrasion, is performed as in smallpox vaccination. Any part of the body may be selected, but we have found the arm most convenient. Walker advises making small incisions in the skin, deep enough to permit absorption, but not deep enough to cause bleeding. A small dental burr may be used, as in the Schick test. The material is placed on the abrasion or incision and allowed to remain one-half hour. If a powder, a solvent should be added after the powder is placed on the skin. If not completely soluble in water, a weak solution of sodium hydroxide, either 0.1 per cent., or N/10 may be employed, as it does not affect the reaction. Walker's studies show that for detecting hypersensitiveness in cases of asthma, hay fever, etc., the cutaneous test is more delicate and yields fewer false positive reactions than the intracutaneous test.

The delicacy of these tests is probably greater than that of any other biological reaction. As has been stated, patients sensitive to extracts of hair of an animal may not be sensitive to the serum proteins and vice versa. Very small amounts of antigen suffice to produce reactions; alkali metaprotein and pepton from hair and dandruff give reactions commonly in dilutions of 1-10,000 and Wodehouse reports one case in which reactions were obtained with dilutions of 1-1,000,000. Clowes reports reaction by means of the ophthalmic test to 0.000,000,05 gram pollen. The fact that positive reactions are found with cutaneous tests in individuals whose serum fails to exhibit antibodies by precipitation, agglutination and complement-fixation tests, is a further indication of the delicacy of the reaction. The accuracy of the reactions is supported by the beneficial results of specific vaccination or desensitization. The treatment is usually by means of subcutaneous injections of the protein to which the patient is sensitive. In cases of sensitiveness to food products, as well as in other cases, patients may be vaccinated by giving the protein by mouth. In either method the amounts are extremely small, and in most instances the course of treatment must be repeated at intervals which may vary from a few months to a year or more. The intracutaneous test appears to be the most delicate in producing local reactions, but unfortunately is more likely to produce confusing traumatic and non-specific reactions to be described subsequently. Details of treatment are given in numerous articles, such as those of Blackfan, Talbot, Goodale, Berger and others in the recent literature.

The Reaction.—This depends to a certain degree upon the particular cutaneous test employed and the sensitiveness of the patient, but in a general way the description applies to all the methods. An urticarial wheal may appear within a very few minutes and may persist for from several minutes to several hours, elevated, firm, pale and itching. Either with or without this preliminary reaction, the passage of a few hours, six, twelve, twenty-four or more, reveals a local area of inflammation about 10 m.m. in diameter, elevated, papular, red, firm and tender. In severe reactions the area may reach a diameter of several centimetres, may be surrounded by an areola of subcutaneous edema, may show fine punctate hemorrhages and may ultimately show vesicles and

crusts. In unusually sensitive individuals the local reaction may be accompanied by systemic manifestations. Less severe but sometimes confusing reactions may appear in the form of pseudo-reactions which are non-specific in nature and probably due to the action of body proteases upon introduced proteins. The reaction to the traumatism from the introduction of the protein may at times be somewhat confusing but in most instances is slight. Certain drugs, such as iodides and bromides, appear to increase the intensity of reactions whether they be specific or non-specific. Iodides are known to reduce the antiferment titer of the blood, and it is possible that the use of these drugs therefore liberates protease and in this way accelerates the non-specific local reaction. The increase of the specific local reactions is probably due to the increase of the non-specific interaction of protease and the introduced protein.

Theories of Cutaneous Reactions.—The appearance of local reactions in hypersusceptibility may be explained according to any of the theories offered for anaphylactic shock. If the mechanism of anaphylaxis involves the formation of poisons these may be concentrated *in situ* because of the irritation produced by introducing the antigen. The irritation leads to a slight local inflammation with its incident vasodilatation and edema. Thus there is a local concentration of antibody, which in reaction with the introduced antigen produces a hypothetical poisonous substance. If the sensitizing substance is within cells, the local contact of antigen in the tissues of the skin explains the local reaction. Similarly the physical theories are adaptable. Stokes, for example, has found that agar will produce a local non-specific reaction. This is probably the result in part of a local loss of balance between ferment and antiferment due to adsorption of the latter by the agar. Similarly any of the physical theories might apply, but the acceptance of the importance of the cells in the reaction, whether physical or otherwise, offers an excellent reason for the early appearance and severity of the local reaction without general manifestations. Cooke, Flood and Coca state that antibodies are not demonstrable in the blood of naturally sensitive persons and therefore emphasize the essential importance of the cells. While agreeing that the cells play a most important part, the experiments of Koessler and the case reported by Ramirez suggest that natural sensitization is of essentially the same nature as anaphylaxis, with marked differences only in the degree of cellular and humoral participation. Therapeutic desensitization of man lasts for a relatively short period of time and differs only in duration from desensitization in experimental animals. In both cases the phenomenon is specific for the antigen employed.

Gay and Force, Gay and Claypole, and Gay and Minaker, in their work with cutaneous reactions in typhoid fever and in the carrier state in meningococcus infections, have expressed the opinion that positive reactions are an indication of resistance on the part of the body against infection by the organisms concerned. Nichols studied the typhoidin test (see page 242) in individuals who had survived typhoid fever and

found that only 75 per cent. of these reacted positively, whereas experience has shown that at least 90 per cent. of such individuals are immune to reinfection. He also pointed out that the immune state following an attack is of much greater duration than is indicated by the typhoidin test. Furthermore, those who have survived typhoid fever or have been vaccinated with bacillus typhosus react positively to paratyphoidin, but it is known that these individuals are not immune to paratyphoid fever. Kolmer and his associates have found no constant parallelism between the presence of positive cutaneous tests and those circulating antibodies, whose presence is indicative of immunity. "The positive anaphylactic skin reaction is, therefore, evidence of infection or sensitization to a particular protein without bearing any direct relation to resistance to infection or reinfection."

Drug Idiosyncrasies.—It is well known in connection with certain drugs, such as morphin, that prolonged use makes it necessary to increase the dose in order to obtain physiological effects. This increase in resistance to morphin is specific, but in the case of chronic alcoholism the individual's resistance to somewhat related substances, such as chloroform and ether, is also increased. Experiment fails to show that this resistance is a state of immunity, and no immune reactions in the ordinary sense of the term have been demonstrated. The use of certain drugs, such as iodoform, iodides, bromides, coal-tar products and quinine, sometimes gives evidence on the part of the patient of a special susceptibility or idiosyncrasy in the form of cutaneous eruptions and more or less severe general symptoms. Both Bruck and Klausner expressed the view that this is an evidence of hypersusceptibility similar to or identical with anaphylaxis. Inasmuch as anaphylaxis is a phenomenon concerning proteins, Bruck offered the hypothesis that the drugs enter into combination with the body proteins, so that a new drug-protein complex of specific character is formed. This protein complex may act as a sensitizer, and upon subsequent injection of the drug there occurs a combination with blood proteins to produce a similar complex which reacts with the sensitizer to produce symptoms. Bruck and Klausner claimed to be able to sensitize guinea-pigs passively with the blood of susceptible patients, so that the animals reacted with the symptoms of anaphylaxis. The autopsies on these animals failed to show the characteristic findings of anaphylactic shock. Cole studied patients sensitive to iodides and to copaiba but failed to obtain results justifying the conclusion that the phenomenon should be included among anaphylactic manifestations. Specific cutaneous reactions to such drugs as quinine and aspirin have been described, and it is maintained that small doses by mouth may desensitize, but no widespread confirmation has been recorded. None of the drugs studied is without some essential toxicity and the idiosyncrasies in some instances, according to Sollmann, "are doubtless due to differences in the strength or constituents of drugs." He further states in regard to increased susceptibility that it "may be due to very rapid absorption, or slow elimination; to the

presence of synergistic substances in the body; or to increased functional susceptibility."

The Tuberculin Test.—In the course of his studies on the treatment of tuberculosis, Koch devised the method of diagnosis which we now speak of as the general tuberculin reaction, in contrast to the local reactions subsequently discovered. It is now known that the introduction of tuberculin into the body may lead to local reactions both at the site of inoculation and in the neighborhood of a tuberculous focus as well as a general reaction which manifests itself in fever, headache and malaise. Numerous methods of preparation of tuberculin for therapeutic and diagnostic purposes have been described, but at the present time the diagnostic methods, in the hands of the majority of workers, depend upon the use of original or old tuberculin of Koch.

For the preparation of this tuberculin now designated as tuberculin O. T. the organisms are grown for six to eight weeks on the surface of 5 per cent. alkaline glycerine broth at 37° C. At the end of this time the entire contents of the flask are sterilized and concentrated to about one-tenth of the original volume by means of a current of live steam and a water bath. The glycerine does not evaporate, and as a result of the concentration constitutes 50 per cent. of the final mass. This is filtered through porcelain and the filtrate employed. Koch subsequently made other preparations, particularly the tuberculin known as T. R. and that known as B. E. The T. R. or tuberculin residue is prepared by growing virulent tubercle bacilli on nutrient glycerine broth for four to six weeks at 37° C. The bacilli are obtained by filtration, dried, and ground in a mortar. One gram is washed with 100 c.c. distilled water, the precipitate is again dried, powdered and repeatedly washed in small volumes of water until no sediment results. The watery extract constituted by this second series of washings, which should not exceed 100 c.c., is preserved with 20 per cent. of glycerine and constitutes the T. R. The bacillus emulsion (B. E.) is prepared by growing the organisms as indicated in the preparation of the original or old tuberculin. The bacilli are obtained by filtration, ground in a mortar and emulsified in 100 parts of distilled water to which is added an equal amount of glycerine.

Numerous other methods of preparing extracts of the tubercle bacillus have been described but are, in essential, modifications of the methods of Koch. At the present time the original or old tuberculin is used most widely.

The General Reaction.—As a general rule, the old tuberculin is put on the market in the form of ampoules of fluid, 1.0 c.c. of which represents 1.0 gram of pure tuberculin. This may be diluted for the actual test. Inasmuch as individual sensitiveness varies considerably, the primary dose should be very small. According to Hamman and Wolman, three classes of patients may be recognized, (a) children, (b) patients who have a slight fever or are not in good general condition, (c) patients in good condition. The smaller doses are given to children and the largest dose to patients in good general condition. Upon this basis the initial dose of old tuberculin should be 0.000,000.1 c.c. to 0.000,001 c.c.; failing to obtain reactions with these doses, subsequent tests may be made at intervals of about a week, increasing the dose each time. Although it is possible to give a maximal dose of 1.0 c.c. of the dilution, it is rarely advisable to exceed 0.05 c.c. The injections should be given under strict aseptic precautions, and appear to be most satisfactory if given at the lower angle of the scapula. They are probably best given in the afternoon, after the patient's afternoon temperature has been taken, so as to avoid the confusion of an unusually high elevation of temperature on the day selected. The reaction appears as a rule in from twenty-four to thirty-six hours. It may appear as late as forty-eight to sixty hours. A positive reaction is indicated by an elevation of temperature of about 2° to 4° C. In

addition there is likely to be headache, malaise, and sometimes a loss of weight. At the site of inoculation there may be pain, tenderness, redness, swelling, sometimes associated with tenderness and enlargement of the regional lymph nodes. The contraindications to the employment of the test include the presence of fever, if fairly high and continued, nephritis, generalized miliary tuberculosis, intestinal ulceration, epilepsy, acute infectious diseases, either during the course of the disease or its convalescence.

The Cutaneous Reaction.—Von Pirquet, who first described this modification of the tuberculin test, originally recommended the use of 25 per cent. solution of the old tuberculin, but subsequently found that the undiluted material is more suitable. He recommends the inner (flexor) surface of the forearm, and suggests the use of three points of scarification about 4 to 5 cm. apart. The skin is cleaned with ether or alcohol before making the abrasions. These may be small scratches with a needle, a knife or with an instrument which he describes as a borer, which has a sharp chisel point and is rotated in order to make a small circular abrasion. A drop of tuberculin is rubbed into the upper and lower abrasions; the middle one remains as a control. In positive cases, the reaction about the point of inoculation is considerably greater than that about the control point. The traumatic reaction in the control may reach a diameter of 3 to 5 mm. in twenty-four hours and then rapidly disappears. The positive reaction usually appears within twenty-four hours, but may be somewhat delayed. Its diameter is ordinarily about 10 mm., but may reach 30 mm. It appears as a red, somewhat tender papule, which in severe reactions may show small vesicles. According to Kolmer, it is not to be interpreted as positive unless its diameter exceeds by 5 mm. that of the control. Occasionally the so-called scrofulous reaction appears, in which papules develop upon other parts of the extremities and the trunk.

The Intracutaneous Tuberculin Tests.—This was described by Mendel and also by Mantoux. For this purpose old tuberculin is injected into the corium in doses whose bulk is 0.05 c.c. Two injections are necessary, one with salt solution and the other with tuberculin. The injection of tuberculin, however, may include three doses of different strengths. The reaction is very similar to that of the cutaneous test. Following a subcutaneous tuberculin test, a similar reaction may appear in the track of the needle.

The Percutaneous Tuberculin Test.—This test was devised by Moro and Doganoff and is frequently spoken of as the Moro skin test. For this purpose 5.0 c.c. of old tuberculin are thoroughly mixed with 5 grams of anhydrous lanolin. This may be preserved for a long time in a light-proof container in the refrigerator and may be obtained on the market in collapsible tubes. About 0.5 gram of this ointment is rubbed into the skin of the abdomen, or breast near the nipple, rather vigorously for one minute. The reaction usually appears within twenty-four hours, but may be delayed from four to six days, and it subsides in three to ten days. It usually appears as a number of small papules reddened at the base. In severe cases the papules may become confluent and vesicles may form.

The Conjunctival Tuberculin Reaction.—This is also referred to as the ophthalmo-reaction and was described independently by Calmette and Wolff-Eisner. Calmette recommended a special aqueous extract of the bacilli but at the present time the test is usually applied with a 1 per cent. solution of old tuberculin. One drop of this solution is instilled into the conjunctiva near the inner canthus. The opposite eye serves as a control. Even in normal individuals the instillation may induce a slight reddening of the conjunctiva within six hours, but the positive reaction appears in from six to eight hours, reaches its height in from twenty-four to forty-eight hours, and then subsides in a few days or a week. The reaction may include simply a slight reddening and swelling of the caruncle, including the neighboring part of the lower lid, may extend over the scleral conjunctiva or may lead to a purulent conjunctivitis. Following the introduction of this test, unfavorable reports were made because of the seeming danger of producing permanent injury to the eye, but Hamman and Wolman state that this danger is not considerable, provided proper precautions are taken in the selection of patients. Diseases of the eye or of the skin near the eye, obvious scrofula in children, and arterio-sclerosis are contraindications. The test should never be applied twice in the same eye, and no stronger solution than 1 per cent. should be employed for the first test. If the first test is negative a 5 per cent. solution may subsequently be employed in the opposite eye.

Theories of the Tuberculin Reaction.—Koch is of the opinion that the amount of tuberculin introduced when added to that already present in the body provides a sufficient amount of toxic substance to produce a definite general reaction. Koehler and Westphal thought that a toxic body is formed in the tuberculous focus by the union of tuberculin and the products of the tubercle bacillus. Marmorek suggested that the tuberculin excited the tubercle bacilli to produce in excess those toxic bodies which lead to fever. Von Pirquet and Schick were the first to suggest that this is a phenomenon related to hypersusceptibility. This conception fits very well the view of the relation of anaphylaxis and immunity which we have indicated above (page 226). Individuals who have markedly active tuberculosis are not likely to react, whereas those who have quiescent or cicatrized lesions almost always react. If the presence of tuberculosis leads to the formation of a sensitizing substance, this can well be absorbed by the cells and be responsible for the local and general reactions. The tuberculin, upon local application, may react with a sensitizing substance in the situation concerned, or upon entrance into the circulation may similarly react with the sensitizing substance in more widely distributed cells, thus producing a general reaction similar in principle to anaphylactic shock. If, on the other hand, the tuberculous process is so active that immune bodies can be found in the circulating blood, combination may be effected in that situation and the cells protected. The study of complement fixation in tuberculosis indicates that this latter assumption is true, namely, that those who have active tuberculosis are more likely to react positively by the complement-fixation test, thereby indicating the presence of circulating antibodies in the active stages of the disease.

Krause has studied this problem extensively, particularly in experimental animals and finds no reason for associating skin hypersensitiveness and anaphylaxis. The anaphylactic state may be induced in animals by parenteral injection of tuberculo-protein, but they do not acquire cutaneous hypersensitiveness. Only by establishing a focus of infection, is it possible to demonstrate a skin reaction. Although during the period of anaphylactic shock an animal may appear to be somewhat less resistant to infection, the state of anaphylaxis produces no alteration in its resistance. Krause is of the opinion that tissue and cutaneous hypersensitiveness and immunity to infection occur under the same conditions, and that one may probably be a function of the other. In the experimental animal the degree of cutaneous hypersusceptibility and immunity parallel each other. He suggests that the local reaction may also appear in the neighborhood of foci of infection and thus aid in walling off the infecting agent. Krause's opinion, based on much admirable work, is worthy of the highest consideration, but in so far as we can determine, it is not in accord with studies of immunity and cutaneous reactions in many other conditions, as pointed out in our discussion of cutaneous hypersusceptibility in general. Petersen considers the tuberculin reaction as a two-phase phenomenon. The primary alteration of the ferment-antiferment balance brings about a

medium favorable for proteolysis in and about the tubercle. Digestion and the liberation of toxic material result and are reflected in the constitutional effects. In the non-tuberculous individual it is probable that the primary serum alterations also occur, but the digestive ferments, finding no focus to attack, liberate no toxic material and no general reaction is elicited. Any agent that brings about a ferment-antiferment ratio favorable for proteolysis will effect a general reaction provided the focus be sufficiently unstable. Conditions such as pregnancy, acute infections, protein shock, in which there is an increase of antiferment, will inhibit the reaction. In late stages of tuberculosis there is also increased antiferment and therefore less marked local reactions but more marked general reactions.

Specificity of the Tuberculin Reaction.—The tuberculin tests have probably been more carefully controlled by autopsy than any of the other clinical tests, and we therefore are able to state with considerable assurance that a positive reaction indicates the presence of tuberculosis in the vast majority of cases, but, on the other hand, gives no very precise information as to the degree of activity of the process. Factors of error are more particularly found in the personal equation of the examiner. Leprosy and actinomycosis, however, may give confusing results. In a very large series of tests, more than 15,000, the percentage of error is very small, varying from 2 to 3 per cent. Negative reactions may appear in markedly active tuberculosis, in the very early stages of the infection, in those small cicatrized lesions of the lung so firmly encapsulated that no absorption takes place, during continued treatment with tuberculin; also during the course of measles, typhoid fever, acute articular rheumatism, pneumonia, diphtheria, pertussis, serum disease and during pregnancy.

Some authors have such confidence in the specificity of the tuberculin reaction that they consider it possible to determine the strain of the organism concerned, but others deny that this delicacy is attainable. The recent work of Petersen would indicate that there is a large non-specific element in the tuberculin reaction. Tuberculous patients may react to the following substances with local and even general reactions: hypertonic salt solution, distilled water, iodides, some colloidal metals, protein split products, etc. Non-tuberculous individuals will tolerate equal doses without reaction. The relation of this type of reaction to the true test has been indicated in discussion of the theories of the tuberculin test.

Utility of the Tests.—At the present time in clinical practice the subcutaneous or general reaction is not very widely employed, because of the prejudice that has been aroused by the possibility of exciting the lesion to renewed activity. Similarly a prejudice exists somewhat unjustly against the use of the conjunctival reaction. Although Hamman and Wolman indicate that the intracutaneous test is the most sensitive, our observation is to the effect that the cutaneous test is most widely employed. It is simple, free from danger, well controlled,

easily read and is sufficiently sensitive to provide all the information that can reasonably be expected to accrue from the tuberculin test.

Luetin Reaction.—Numerous attempts were made following the announcements of the Von Pirquet cutaneous tuberculin test, to devise a similar test for syphilis. It was found, however, that extracts of normal organs produced the same effects as those from syphilitic organs. It was not until Noguchi cultivated the *treponema pallidum in vitro* that a preparation of the causative agent could be prepared. Noguchi prepared a suspension of the organisms together with the ascites-kidney agar upon which they were grown. Cutaneous reactions were unsatisfactory, and it was found necessary to make the test by intracutaneous injection of the material. The reaction appears in papular or pustular form in from twenty-four to forty-eight hours or later. It was found by Sherrick that patients receiving potassium iodide give positive reactions and by Cole and Paryzek that similar reactions follow the administration of bromides. Although Noguchi found that injection of the culture medium without the organisms did not produce reactions, Stokes as well as Kolmer, Matsunami and Broadwell were able to produce reactions by injecting agar. Although Noguchi and others reported high percentages of positive reactions in known syphilitics, yet in the hands of some workers the number has been only about 50 per cent. The test is not widely employed and apparently gives no information that cannot be obtained equally well or better from the Wassermann test. It has been suggested that the luetin test may be of value in late syphilis, where the Wassermann test is negative, but the large non-specific element of this skin reaction does not tend to place much reliance upon the test.

Cutaneous Reactions in Typhoid Fever.—Several of the earlier studies on this subject were concerned with reactions in the conjunctiva. Chatemesse and also Austrian were able to obtain positive ophthalmoreactions in a large percentage of cases of typhoid fever and practically no positive reactions in other individuals. Although Kraus could not obtain skin reactions, Zupnik and also Floyd and Barker obtained encouraging results. Gay and Force have employed a substance which they name typhoidin, prepared from *bacillus typhosus*, according to the method employed for the preparation of old tuberculin. The preparation was modified subsequently by Gay and Claypole. The typhoidin is applied in abrasions of the skin as with the cutaneous tuberculin test. These investigators found a high percentage of positive reactions in individuals who had recovered from typhoid fever as well as those who had been vaccinated and recommend it as a method for determining the presence of immunity to typhoid fever. Kilgore has studied the test clinically and finds that the test is unreliable because of unavoidable variations in the application of the test, indefiniteness of the readings and the large non-specific element in the reaction.

Cutaneous Reactions to Gonococcus Infections.—These reactions are particularly applicable to deep-seated and chronic infections with the gonococcus. Irons found local and general reactions following the

subcutaneous injection of gonococcal vaccine and subsequently prepared a glycerol extract of the organism for cutaneous tests. He instituted controls with equal quantities of glycerol and obtained distinctly encouraging results even to the point where one strain of organism produced stronger reactions than other strains.

Cutaneous Reactions to Meningococcus Infections.—Recently Gay and Minaker have employed the intracutaneous reaction for the detection of meningococcus carriers. They prepared a salt solution emulsion of carefully washed and thoroughly dried cultures of five strains of meningococcus and injected 0.000,006 gram of the dried powder in a total volume of 0.05 c.c. They obtained reactions in 64.5 per cent. of known carriers and 26.4 per cent. in individuals known not to be carriers. They do not think that the reaction serves any important purpose in diagnosis but suggest that it may indicate a systemic reaction and possibly a certain degree of acquired resistance to the organism.

Cutaneous Reactions to Pneumococcus Infections.—Earlier investigations with salt-solution extracts were not particularly satisfactory in regard to the early diagnosis of pneumonia, although after the crisis reactions were obtained. Weiss and Kolmer prepared a solution of Type I pneumococci in sodium choleate which they designate *pneumotoxin*. The test is performed by intracutaneous injection. By careful study of animals, on the basis of both gross and microscopic examination of the site of reaction, as well as of human patients with pneumonia, they obtained distinctly encouraging results during the course of the disease and state that although the test does not seem to be of distinct value in differentiating the type of organism concerned, yet it may aid in differential diagnosis between appendicitis, tuberculosis and pneumonia.

Cutaneous Reactions to Vaccine Virus.—Jenner noted that in certain individuals who had previously been vaccinated against smallpox, a second vaccination might produce a local reaction which did not go on to produce vaccinia. This has been observed by numerous investigators since then and Force has given the subject close study. For this purpose Force produced three abrasions on the arm, into two of which vaccine virus was rubbed and made observations at the end of twenty-four, forty-eight and seventy-two hours. "If either of the vaccinated spots showed an areola of 5 mm. or over (with or without papule) at the end of twenty-four hours, which areola (or papule) had decreased at the time of the seventy-two-hour observation, it was considered a *reaction of immunity* due to the presence in the blood of the individual of antibodies against vaccine virus." "If either of the vaccinated spots showed an areola at the end of twenty-four hours which developed into a small vesicle, maturing on the fifth or sixth day and then rapidly subsiding the reaction was considered a *vaccinoid*," a condition in which it is supposed antibodies are not present but are rapidly formed because of a previous vaccination, thus leading to the small size and rapid subsidence of the vesicle. "If there was no change until the third day, and then a small areola began to form, the case would be

vaccinia." This description indicates the changes that may appear following an uninfected vaccination with smallpox virus. There apparently occurs, following smallpox and vaccinia, an altered state which determines these local reactions, but the interpretation offered by Force that some of these reactions are immune reactions still lacks satisfactory confirmation and is not consistent with other studies of cutaneous reactions (see page 237).

Cutaneous Reactions in Glanders.—The Mallein test devised by Kelmann and Kelming is widely employed in veterinary practice, either in the form of subcutaneous injection which produces a general reaction as is the case with tuberculin, or in the form of conjunctival test which produces local and often general reactions.

Other Cutaneous Reactions.—As can very well be understood the encouraging results with such a large number of skin reactions has led to the investigation of similar tests in other diseases and the reaction has been applied in leprosy, sporotrichosis, hyphomycetes infections, pregnancy, canine distemper and numerous other conditions. The Schick test for diphtheria is not to be included among the skin reactions indicating hypersusceptibility, for, as has been shown previously, this test depends upon the presence or absence of antitoxin in the circulating fluids of the body.

CHAPTER XII

DEFENSIVE FERMENTS

INTRODUCTION.

SPECIFICITY OF FERMENTS.

IMMUNE FERMENTS.

FERMENTS IN THE BLOOD.

FERMENT-ANTIFERMENT BALANCE.

ANTIFERMENT.

THE ABDERHALDEN TEST.

Introduction.—The relation of ferments to immunity and anaphylaxis has long been the subject of discussion. In the chapters on special immune bodies we have discussed the similarities and differences between ferments or enzymes and antibodies. Special consideration has been given to certain phases of ferment activity, particularly in the chapter on Cellular Resistance and that on Hypersusceptibility. Apparently the first work to prove that digestion takes place outside the intestinal tract was that of Hammersten, who showed in 1885 that washed leucocytes increase the solubility of fibrin. This was followed by more comprehensive studies on cellular ferments as have been previously outlined (page 167). In addition to those ferments which exist in the cells, ferments have been discovered in the blood and other circulating body fluids. Therefore, we may classify the ferments as intracellular and extracellular. The scope of this book is too limited to permit of any general discussion of ferments as a group and the reader is referred to the sections on this subject in Wells' "Chemical Pathology." Many of the earlier workers assumed that ferments in the body fluids are derived essentially from the leucocytes. A recent study of considerable significance is that of Boldyreff. He maintains that the glands of the alimentary canal, with the exception of those of the stomach, are not at rest between the digestive periods and that they exhibit a periodic function. As a result of this periodicity, secretions are discharged into the empty intestine from which they are absorbed and at times are demonstrable in the blood. Van Calcar claims that the leucocytes are incapable of producing their own ferments and that these ferments are derived from special glands. He found that extirpation of the stomach is followed by a decrease or absence of that ferment of the leucocytes which acts best in acid medium and that extirpation of the pancreas similarly is followed by a loss of tryptic powers on the part of the leucocytes. Abderhalden believes that invertin also is derived from the intestinal glands. This conception would indicate that the appearance of ferments in the circulating body fluids is to be regarded as a mobilization of ferments from the cells which formed them.

Specificity of Ferments.—It is well known that the body ferments act specifically upon certain chemical substances, as exemplified by the

digestion of starch by amylase and of protein by pepsin. The question as to whether or not specificity in the immunological sense can be demonstrated has been the subject of much discussion. Claims have been made for specificity of ferments not only in regard to animal species but also in regard to specificity for the cells of particular organs. The chief proponent of the specificity of ferments for cells and proteins is Abderhalden. He was stimulated to this view by the work of Schmorl and others, who demonstrated that during pregnancy fragments of the syncytium of the chorionic villi often enter the circulation and by the claims of Weinland that specific reducing ferments are produced following the parenteral introduction of cane sugar. Abderhalden thereupon examined the blood serum of pregnant animals and found that the serum contained a ferment capable of splitting placental pepton into amino-acids and of digesting coagulated placental tissue into pepton, polypeptids and amino-acids. These decomposition products are diffusible and also alter the axis of optical rotation of the mixture. The detection of the diffusible products of protein decomposition was made by means of "ninhydrin" or triketohydrindenhydrate, which reacts with alpha amino-acids so as to produce a blue or violet color. The practical application of a test of this sort is obvious and the method has been employed to detect specific ferments in pregnancy, in carcinoma, in sarcoma, in diseases of the brain, of the eye and of numerous other organs. Practical experience with the test, as well as further scientific study, has made it seem probable that the specificity claimed by the Abderhalden school does not exist. This will be further discussed in connection with the Abderhalden test.

Immune Ferments.—Numerous investigators have published reports indicating that the parenteral introduction of special substances leads to production of special ferments or at least to an increase of preëxisting ferments in the form of a mobilization. Delezenne reported in 1900 that the injection of animals with gelatine produces a blood serum capable of liquefying gelatine. Weinland in 1907 showed that although normal dog serum cannot reduce cane sugar the immunization of a dog by several injections of cane sugar leads to the formation of a ferment capable of reducing cane sugar *in vitro*. Similarly, immunization with edestin produces a serum capable of splitting this substance. The more recent investigations of the subject would make it appear that the immunization leads rather to mobilization of non-specific ferments than to the production of a specific immune body.

Ferments in the Blood.—Wells states that the blood contains diastase, glucase, lipase, thrombin, rennin and proteases. In addition, the blood possesses oxidizing properties due presumably to the presence of oxydase, peroxydase and probably also due to catalase. The proteases have been given particularly careful study. Petersen divides these ferments into the leucoproteases, serum proteases and serum peptidases. The leucoproteases include (a) an active ferment operating in alkaline medium and capable of digesting native protein to the proteose stage, (b) an active ferment capable of operating in acid medium with

a similar range of activity, and (c) an ereptase active in both acid and alkaline media and capable of splitting partially hydrolized proteins into amino-acids. Of these ferments only the ereptase is able to act in the presence of blood serum and tissue fluids because the others are inhibited by the activity of antiferment constantly present. The serum protease is a polyvalent trypsin-like ferment active in neutral, in slightly acid, or in slightly alkaline media; it is completely inhibited by the antiferment of the circulating fluid. It becomes active only when the antiferment is removed and is capable of digesting native protein to the amino-acid stage. It is present in fairly large amounts in sera of the lower animals, but is found in only small quantities in human serum. The serum peptidase is a polyvalent ferment which operates in the same type of media as the protease; it is present in normal human serum in small amounts, is not inhibited by antiferment and digests partly hydrolized proteins to the amino-acid stage. Since the toxic fractions of proteins are principally in the form of proteoses and pepton, the peptidase apparently is the most important ferment in destroying such toxic bodies.

The importance of esterases in the blood is not at all clear. It is known that lymphocytes contain a lipase, and it has been suggested that the accumulation of these cells about tuberculous foci may indicate the importance of this ferment in breaking down the waxy capsule of the bacilli. Jobling, Petersen and Eggstein recommend the following methods for the determination of serum protease and serum esterase (*Journal of Experimental Medicine*, vol. xxii.).

"The technic for proteases is as follows: The clear hemoglobin-free serum is measured with an accurate 1.0 c.c. pipette into a rather wide test-tube (about 18 mm.). To the tube 0.5 to 0.75 c.c. of chloroform is added and the tube is sharply shaken, at intervals, until a milky emulsion is formed. We prefer chloroform because the emulsion is more stable than with ether or other lipid solvents. A control tube is inactivated at 60° C. for thirty minutes and a drop of toluol is then added in place of chloroform. Both tubes are then incubated over night (fifteen to sixteen hours at 37°). In the morning about 1.0 c.c. of a mixture of 10 per cent. acetic acid plus 20 per cent. salt solution is added, and the tubes are then gently warmed in a water bath until the chloroform has been evaporated. About 2 or 3 c.c. of distilled water are then added slowly and the tubes boiled for at least ten minutes. The coagulated protein is filtered off by means of hard filter paper, previously moistened, the filtrate being permitted to filter directly into the large tubes used for oxidizing. The tubes are then oxidized and Nesslerized according to the usual Folin method, the readings being made against varying dilutions of the 1 mg. standard, so that test readings are made against standard of apparently equal color."

"Serum esterase has been determined as follows: To 1.0 c.c. of the serum, 1.0 c.c. of neutral, redistilled ethyl butyrate and 0.5 c.c. toluol are added, the volume being brought to 10.0 c.c. with physiological salt solution. The flasks are then shaken 100 times and incubated for four hours; 25 c.c. of neutral 95 per cent. alcohol are then added to each flask and the acidity which has developed is titrated with N/50 sodium hydrate (alcoholic) to a faint pink with phenolphthalein. After deducting the proper controls, i.e., serum alone, ethyl butyrate alone, etc., the esterase index is expressed in terms of c.c. of N/100 sodium hydrate used to neutralize the acidity developed by 1.0 c.c. of serum from 1.0 c.c. of ethyl butyrate."

Ferment-Antiferment Balance.—The activity of various ferments in the body is probably effective in various degrees at all times, and this activity probably plays a certain part in normal metabolism. Cer-

tain of the ferments become active only when suitable material is presented, as is the case with the serum peptidase; others operate only when the surrounding medium reaches suitable reaction; still others operate only if the antiferment content is sufficiently reduced. Therefore, the preservation of the body tissues against destructive action of ferments and the normal processes of metabolism depend in considerable part upon the neutralizing activity of antiferments.

Antiferment.—Certain investigators have reported the production of specific antibodies following the injection of ferments. Morgenroth claimed to have produced a specific antirennin, Sachs and Achalme an antipepsin and an antitrypsin, Schultze an antisteapsin and an antilactase, Gessard an antityrosinase and Moll an antiurease. The recent studies of antiferments, however, indicate that inhibitory activity is not specific and this subject has been contributed to particularly by Jobling and his collaborators. They are of the opinion that antiferment activity depends upon the highly dispersed unsaturated lipoids of the serum and lymph and that the titer varies with the amount of lipoids, their dispersion and chemical structure. In studying antitrypsin, they found that the inhibitory substances are of the nature of soaps and that the ability to inhibit ferment activity depends upon the degree of unsaturation of the carbon bonds in the fatty acid. They made soaps from olive oil, cod-liver oil, linseed and other oils and found that these soaps inhibited the action of trypsin and leucoprotease. They determined further that extraction of the blood serum with such fat solvents as chloroform and ether removes the antitryptic activity. Soaps prepared from the extracts restored the antitryptic activity. The serum residue, after extraction, was found to be highly toxic for guinea-pigs. If, however, the soap prepared from the extract were added to the residue, the toxicity was neutralized. Jobling and his collaborators attributed the toxic action of the serum residue to (a) alteration of the mechanism of intravascular coagulation, (b) exposure of native serum proteins to the action of ferments and (c) the resulting formation of toxic split products. These workers isolated unsaturated fatty acids from tubercle bacilli and found that when these were saponified they inhibited the action of trypsin but lost this power when saturated with iodine. They were able to obtain similar soaps from caseous lymph-nodes and suggest that the soaps prevent the activity of ferments which would normally digest the necrotic material. This failure of digestion leads to the formation of the partly-digested and fatty substance which is spoken of as the caseation necrosis.

The antiferments are greatly augmented in certain diseases, such as acute infections, carcinoma, cachexias in general, anaphylactic shock, certain degenerative changes of the nervous system and in pregnancy. Jobling explains the crisis of pneumonia as being due to an alteration in the ferment-antiferment balance; that there is a decrease in the antiferment with a corresponding mobilization of protease, an increase in the serum lipase with a resulting decrease in the non-coagulable nitrogen and proteoses of the serum.

Determination of Antiferment in Blood Serum.—The determination of antitrypsin by the Fuld-Gross method is satisfactory for this purpose. This requires in addition to blood serum taken preferably in the morning before the patient's breakfast, solutions of casein, acetic acid, and trypsin. The solution of casein is made by dissolving 1 gram casein in 100 c.c. N/10 NaOH with the aid of slight heating; the solution is neutralized with N/10 NaCl and made up to 500 c.c. with 0.85 per cent. NaCl. The acetic acid solution is made by mixing 5.0 c.c. acetic acid with 45.0 c.c. alcohol and 50.0 c.c. water. The trypsin solution is made by dissolving 0.5 gram trypsin (Grübler) in 50.0 c.c. 0.85 per cent. NaCl and 0.5 c.c. normal soda solution; this is diluted ten times with saline. The patient's serum must be fresh and should be diluted with salt solution so as to make a 2 per cent. solution.

The trypsin is titrated as follows: Place in a series of test tubes 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 c.c. of the trypsin solution. Add 2.0 c.c. of casein solution to each tube, shake and incubate for one-half hour at 50° C. Add three or four drops of the acetic acid solution to each tube and note the precipitation (cloudiness) which appears in the course of a few minutes. The tube which remains perfectly clear contains enough trypsin to digest 2.0 c.c. of the casein solution. For testing the antitryptic content of the serum add 0.5 c.c. of the 2 per cent. solution of serum to each of six small test tubes. Then add to each tube in series, increasing amounts of the trypsin solution, beginning with the largest dose that completely digested the casein, and increasing in each tube by 0.1 c.c. Add 2.0 c.c. of casein solution to each tube and make up to equal volumes with normal saline. Shake and incubate for one-half hour at 50° C.; then add three or four drops of the acetic acid solution to each tube and observe as before. The amount of trypsin which is inhibited by the serum is determined by the lack of complete digestion, as shown by the acetic acid precipitation. A control series should be set up with the pooled sera of normal individuals. Jobling and his associates made the test somewhat more accurate by filtering after the incubation and then determining quantitatively the non-coagulable protein nitrogen.

The Abderhalden Test.—In the discussion of the specificity of ferments, we pointed out that Abderhalden had assumed that the entry of cellular and other proteins in the circulation could lead to the formation, or increase, of ferments which have as their specific character the property of digesting the antigenic protein. The test is based fundamentally upon a mixture of serum and antigenic substance and the determination of the formation of diffusible protein products. He regarded the ferments as protective, inasmuch as they could break down and aid in the elimination of substances essentially foreign in nature. The technic of the test has been carefully reviewed by Bronfenbrenner in Vol. I of the *Journal of Laboratory and Clinical Medicine*, and we call particular attention to certain modifications that have been offered by Retinger in Volume XXII of the *Archives of Internal Medicine*. The following brief description of the test is given in order to provide an outline of the general principles.

The materials essential for the test are the serum or plasma of the patient, the substratum, dialyzing tubes and flasks, carefully distilled water, clean test tubes and ninhydrin. The blood is withdrawn before the patient's breakfast in order to obtain blood at a time when no dialyzable products of intestinal digestion are present. It may be taken into paraffin-coated centrifuge tubes for the preparation of plasma or may be allowed to clot and the serum centrifuged so as to be absolutely clear. The substratum is the material to be digested. As a rule, the tissue is cleared of connective tissue in so far as possible, is perfused with salt solution and subsequently washed several times with distilled water until it is absolutely free from blood. It is then placed in a suitable container, coagulated by boiling, and repeatedly washed with boiling water until the fluid gives no ninhydrin test. It is then

preserved under toluol. The dialyzing thimbles are especially prepared for work of this kind. They are kept in distilled water for at least a week and are carefully tested before use so as to be sure that protein does not pass through and also to be sure that pepton will pass through. For the actual test a dialyzing thimble is placed in a clean, dry, sterile Erlenmeyer flask. About 0.5 gram of dried substratum is placed in the bottom of the thimble and 1.5 c.c. of serum then introduced. The thimble is withdrawn, closed at the top by means of a forceps and the outside washed carefully with sterile water so as to remove any adherent protein. The thimble is then replaced in a flask containing about 20 c.c. sterile distilled water. The contents of the thimble and the water in the flask are covered with toluol and the flask incubated for sixteen to eighteen hours. The dialyzate is examined by means of the ninhydrin test. For this purpose, 0.2 c.c. of 1 per cent. ninhydrin solution is placed in a clean dry test tube and 10.0 c.c. of the dialyzate added, the mixture boiled for one minute and the color observed. The development of a blue or violet color indicates the presence of diffusible protein products and constitutes a positive test. Proper controls of all the reagents are essential.

Since the earlier work of Abderhalden appeared, numerous articles have been written and there has been much discussion concerning the alleged specificity of the reaction. The protective ferments of Abderhalden are assumed to possess the property of directly digesting the antigen and the appearance of the products of such direct digestion constitutes the fundamental principle of the Abderhalden test. Stephan, Hauptmann, Bronfenbrenner and others have shown that these ferments lose their activity after heating to 58° C. for one-half hour, but they can be reactivated by the addition of fresh serum. This suggests a parallel with the activity of complement and amboceptor, but Frank and Rosenthal point out that in hemolysis there is no indication that the action of complement is accompanied by proteolysis. Therefore, although the ferment may be reactivated after heating, this does not necessarily indicate that it is of the nature of an amboceptor or other immune body. Flatow, Plaut and others have reported that positive results can be obtained by the manipulation of material and that positive or negative reactions can thus be found with almost any serum. De Waele found that he could demonstrate a digesting substance within a few minutes after the parenteral introduction of foreign protein, a time interval too short for the production of specific ferments. Heilner and Petri regard this, however, as a sort of mobilization of ferment and not the result of new formation. Bronfenbrenner found that the serum of highly immunized animals failed to digest the protein used for immunization. He determined, however, that such a serum gave a positive Abderhalden test and with his collaborators has demonstrated that the dialyzable substances do not originate from the substratum. He showed also that the ferments responsible for the cleavage of protein during the reaction are not specific. Positive results with placenta are to be obtained with the serum of males as well as of females, but the protein digested is that of the serum. The work of Jobling and his collaborators favors the view that proteolytic activity of the serum is not specific. Plaut, Bronfenbrenner and others found that positive Abderhalden tests may be obtained by the use of kaolin, starch, barium sulphate and chloroform, all of which probably absorb the inhibiting substance or antiferment of the blood. Van Slyke and his associates,

by means of determining the amino-nitrogen, found that practically every serum shows some degree of protein digestion when incubated with placental tissue. Van Slyke's methods are so accurate that it seems probable that the ninhydrin tests with dialyzates must vary considerably, depending upon the amount of dialyzable substance which may pass through any given thimble. Elsesser worked with the purified vegetable proteins of Osborn and found that at best the specificity of the reaction is less than that of anaphylaxis and that there are many non-specific results. Boldyreff found that the ferments act not only upon placental proteins but also upon other varieties of protein; he believes that the method is excellent for detection of proteolytic enzymes in the blood but as a distinctive sign of pregnancy it is useless. Against these views are the recent results of Retinger, who claims that it is not only possible to demonstrate lesions of the brain by this test but further to define within fairly small limits the localization of the lesion. It may be that with further modifications a test of some clinical value can be developed upon the basis of the Abderhalden test. At the present time, there is little reason for accepting the conception of specific ferments and the test has been entirely discarded in many laboratories.

APPENDIX A

THERAPEUTIC EMPLOYMENT OF BLOOD SERUM

INTRODUCTION.

SERA PREPARED BY USE OF BACTERIA OR BACTERIAL EXTRACTS.

ANTI-STREPTOCOCCUS SERUM.

ANTI-MENINGOCOCCUS SERUM.

ANTI-PNEUMOCOCCUS SERUM.

ANTI-CHOLERA SERUM.

ANTI-ANTHRAX SERUM.

ANTI-PLAGUE SERUM.

ANTI-BACTERIAL SERUM FOR DIPHTHERIA CARRIERS.

ANTI-GONOCOCCUS SERUM.

ANTI-TUBERCULOSIS SERUM.

ANTI-TYPHOID SERUM.

AUTO-SERUM THERAPY.

GENERAL USES.

SYPHILIS.

HUMAN IMMUNE SERUM.

SERUM THERAPY IN INFECTIONS OF UNDETERMINED ETIOLOGY.

INTRODUCTION.

ANTI-POLIOMYELITIS SERUM.

ANTI-HOG-CHOLERA SERUM.

THERAPEUTIC USE OF NORMAL SERUM.

THE development of immunology has resulted in extensive study of the treatment of disease by sera prepared according to a variety of methods. We have considered in other chapters the value of certain sera, more particularly those which possess a demonstrable content of antitoxin. In this chapter there is presented a brief statement as to the methods of preparation and use of other types of sera with the idea of illustrating how widely this form of therapeutics has extended and the principles upon which the methods are founded. Certain of these sera have given excellent results, but others have failed utterly and still others are yet in the stage of experiment and investigation. The judgment as to the value of many of the sera rests upon statistical evidence collected on a clinical basis. The use of man for investigation intrudes into the results obtained a wide variety of sources of error, many of which can be excluded in investigations upon the lower animals. Differences in hygienic surroundings, conditions of exposure, presence of diseases other than that treated, differences in weight, age and sex must all be considered. The stage of increase or decrease of the epidemic must be included in the final judgment since the virulence of infections is likely to be greater at the beginning of an epidemic than during its decline; this may be due to exhaustion of the causative agent, but is more probably accounted for in that the less resistant individuals succumb early in the epidemics and the more resistant are attacked subsequently. The factor of error in random sampling must be calculated as closely as possible and it must be recognized that the greater the number of cases studied, the more conclusive are the results. The

investigator is always actuated by the hope that the particular method he fosters will be of value in the alleviation of human disease, and this fact may determine a subconscious selection of cases and perhaps, equally subconscious, somewhat superior nursing and better care of the cases under special treatment than of the controls. Thus the analysis of statistical evidence must be made with rigid consideration of the various factors of error. Minor differences in percentages of cure or of improvement may be within carefully computed factors of error and still not take sufficiently into account a considerable factor of error resulting from our ignorance of the intricacy of biological phenomena.

Immune sera for therapeutic purposes have been prepared by the injection of bacteria, their toxic or non-toxic extracts, or by combinations of these substances. Some of these sera exhibit a variable content of antibodies of the first, second or third order of Ehrlich. The most important laboratory test, however, appears to be the protective value of the sera in preventing infection in animals or their curative value after the infection is established. There is no necessary parallel between the content of special antibodies and the protective or curative value, except in the case of antitoxic sera. Thus a serum may exhibit a low agglutinin or bacteriolysin content and yet protect animals when used in extremely small amounts. The converse is also true, namely, that relatively high content of agglutinin or bacteriolysin does not necessarily presuppose a great capacity for protection. Furthermore, it cannot be assumed positively that because animals are protected or cured, the serum will be of equal value in human medicine; hence the necessity for carefully studied experiments on man.

Not only have immune sera been employed, but many attempts at treatment of disease by means of normal sera have been made. This procedure is based in part upon the principles of non-specific immunological treatment which have been previously discussed. Such sera may be obtained from man, horse, goat, ox or other animal. In the treatment of certain hemorrhagic disease the purpose of the serum may be physiological rather than immunological, inasmuch as the serum is believed to provide certain essentials for the process of clotting which the patient provides in insufficient amounts or not at all.

In the following discussion it will be noted that there are first taken up those sera prepared by immunization with bacteria; second, those prepared by immunization with bacterial extracts, either with or without the bacterial bodies; third, treatment with the patient's own serum; fourth, treatment with sera from convalescent human cases; fifth, specific serum therapy in diseases of unknown origin, and finally, treatment by normal sera.

SERA PREPARED BY USE OF BACTERIA OR BACTERIAL EXTRACTS

Anti-streptococcus Serum.—The protection afforded by the use of streptococcus immune serum is still problematical. The reason for this lies partly in the fact that there are several different types of streptococci concerned in human infection. Some of the strains occur frequently and

others only rarely. It is, therefore, advisable to determine as soon as possible the type of the organism, and then combat it with its special antiserum. Havens succeeded in dividing these organisms into three distinct groups by means of cultural and immunological examination and found that an immune serum can be produced for each of the three groups. The serum is specific for its own group and protects mice against infection with homologous organisms, but furnishes no protection against infection with organisms from the other groups. From this work it is evident that the utilization of specific sera is of paramount importance in the treatment of streptococcus infections. The oldest serum is that of Marmorek. This serum was produced by immunization with a strain which was made highly virulent by animal passage and the serum was found to be protective experimentally when administered twelve to eighteen hours before the bacteria were injected. This serum was used in erysipelas, puerperal septicemia and scarlatinal angina with favorable results. Lenhartz, Baginsky, Zangemeister and others, however, failed to obtain definitely good results with anti-streptococcus sera. Sera were later produced by Aronson and Tavel, Van de Velde, Meyer, Ruppel, Menzer and Moser for use in puerperal sepsis, scarlatina, erysipelas and acute articular rheumatism. In puerperal infection a fresh polyvalent anti-streptococcus serum should be given daily in intravenous doses of 30. c.c. until marked improvement occurs. These cases are usually slow in improvement, but the results so far obtained seem to be encouraging. It is, however, of the greatest importance to introduce serum treatment at the earliest possible moment. In scarlatina Escherich found that if the serum be used on the first and second days of illness recovery of the majority of cases is likely to occur. Axenow, in fact, believes that it is the only means to ward off a fatal outcome. In erysipelas and acute articular rheumatism the results have been at variance. Park states that the injections should be made before the infection has become advanced and before the streptococci have acquired an increased resistance to the serum antibodies and ferments. The repeated local bathing of exposed infected tissues with the serum seems to have a beneficial result beyond that exercised by a non-specific serum. The action of anti-streptococcal sera is largely due to its opsonic powers. The hope for effective serum therapy in streptococcus infection is at present based on the new methods of serologic classification of the organism and further laboratory and clinical study is highly desired.

Anti-meningococcus Serum.—In 1906 Jochmann for the first time treated cerebrospinal fever with serum of horses immunized to several strains of meningococci. This serum was highly agglutinative, somewhat bactericidal, but not antitoxic. The death rate among the treated cases was 27 per cent. as compared to 53 per cent. among the non-treated cases. In the earlier work Jochmann administered the serum subcutaneously but later advised its use by the intraspinal method. Almost simultaneously Flexner and Jobling carried out extensive work on monkeys. They demonstrated that the most beneficial effect of the

serum follows intrathecal administration, and in 1907 successfully applied serum treatment of the disease during an epidemic in Akron, Ohio. The following table, taken from Worster, Drought and Mills Kennedy, "Cerebrospinal Fever," London, 1919, gives the results obtained by several investigators.

Author	No. of treated cases	Serum used	Serum treated mortality	Cases not treated with serum mortality
Flexner ...	1294 (collected)	Flexner	30.9%	70%
Netter	100	Flexner	28.0%	49%
Dunn	40	Flexner	22.5%	70%
Robb	300 ±	Flexner	30.0%	72%
Dopter ...	402	Dopter	16.4%	65%
Levy	165	{ Kolle and Wassermann }	18.8%	52%
Steiner ...	2280 (collected)		37.0%	77%
Schoene ..	30	Jochmann	30.0%	53%

Although many English investigators have been successful in the use of anti-meningococcus serum, several experienced men have advised against its use. This is largely because of the fact that Gordon, Ellis and others have demonstrated several types or groups of the meningococcus, and it is believed that sera should be prepared against each type in order to obtain the best results. Rolleston has compiled the following table of results with various types of anti-meningococcus sera:

Brand of serum	Mortality	Recoveries
Flexner	22.3 per cent.	77.7 per cent.
Gordon	18.7 per cent.	81.3 per cent.
Pasteur Institute	44.5 per cent.	55.5 per cent.
Burroughs-Wellcome ...	33.3 per cent.	66.7 per cent.
Mulford	50.0 per cent.	50.0 per cent.
Lister Institute	54.5 per cent.	45.5 per cent.

Gordon's and Flexner's sera have so far given the best results. It is advisable to test the agglutinative power of a serum prior to its use, using a strain freshly isolated during the epidemic. The serum should agglutinate the organism in a dilution of at least one to five hundred. The lack of a definite potency standard makes it impossible to judge accurately the value of any given serum. As in diphtheria and other diseases, the early use of serum is of the greatest importance. Flexner found that if the serum was given in the first three days the mortality was 18 per cent., if given from the fourth to the seventh day it was 27 per cent., and if given later 36.5 per cent. Similar figures were obtained by Rolleston, Gray, Robb and Worster-Drought. If necessary, the injections should be repeated. According to Park, it is advisable to give not less than four daily injections unless the case is already convalescent when it comes under observation. If the organisms or symptoms do not disappear, the injections of 10 c.c. to 25 c.c. of serum should be continued for many days. Finally, as a result of army experience, Herrick believes that the disease is in most if not all cases a general bloodstream infection with secondary meningeal involvement and therefore advises the use of large doses of anti-meningococcus serum intravenously as soon as the diagnosis is made in addition to

intrathecal injections. The results so far obtained seem to be better than when intraspinal injections alone are used. Frich also recommends that all patients with positive signs and symptoms be given both intraspinal and intravenous injections of serum. Large doses of serum, both intravenously and intraspinally at frequent intervals apparently do no harm, lower the mortality, prevent serious complications and shorten the period of convalescence.

Anti-pneumococcus Serum.—Washburn, Mennes, Pane and numerous other early investigators attempted to produce anti-pneumococcus sera for the treatment of man, but their results were irregular and not encouraging. An important advance in the production of anti-pneumococcus serum was made when Neufeld and Händel in 1909 pointed out that pneumococci can be divided into various immunological groups, and that no curative properties can be expected from a given serum unless it is homologous for the type that causes the infection. This work has been confirmed and extended by Dochez and Gillespie, Cole, Lister and many others. At present we recognize four groups. Groups I and II are immunologically distinct groups, Group III is that of the streptococcus or pneumococcus mucosus, and Group IV a heterogeneous group of pneumococci which cannot be classified under the other three groups. The following table, taken from Park ("The Practical Application of Serum Therapy," Transactions of the Congress of American Physicians and Surgeons, 1916, x, 118) gives the group-incidence and mortality:

Type	Number		Per cent. incidence		Per cent. mortality		University of Penna. Hospital, Richardson		
	Cole	Longcope	Cole	Longcope	Cole	Longcope	No.	Per cent. incidence	Per cent. mortality
I.	78	*(13)	33	*(23)	25.0	*(12.5)	60	31	30
II.	75	(11)	32	(21)	29.0	(72.7)	39	20	25
III.	22	(7)	9	(14)	45.0	(85.7)	13	6	50
IV.	48	(21)	20	(40)	12.5	(23.8)	83	43	12
Other bacteria	14	...	6

* Presbyterian Hospital, Longcope.

From this table it appears that about 30 per cent. of the cases of pneumonia and about one-third of the total deaths from the disease are caused by Type I pneumococci. In the United States Cole claims that 75 per cent. of all pneumonia cases are caused by Types I, II and III, and 25 per cent. by Type IV. Lister, in South Africa, finds Type IV very common among the negroes in the Rand. So far only Type I and Type II sera have given encouraging results. The antigenic value of Type III pneumococcus is exceedingly low, and that of Type IV variable. From the more recent work of Raphael it would appear that sera produced against various strains of pneumococci are in a sense strictly monovalent and also that only virulent pneumococci are sufficiently antigenic to produce antisera of distinct value.

The sera against infection with Type I organisms have been used extensively and appear to have given especially good results. The

Type II antiserum, however, is much less efficacious and thus far its therapeutic value is questionable. The Types III and IV antisera have no clinical value. Dochez reports sixty-five cases treated with Type I serum, with a mortality of 6.6 per cent., as compared with a mortality of 25 per cent. in Type I cases not treated with serum. Type II cases treated with serum have a mortality of 25 per cent. as compared with 61 per cent. without the use of specific sera. When patients are treated early, they do well, and large doses of serum should be given as soon as the type of infection has been determined. Cole advises an intravenous injection of 80 to 100 c.c. of serum diluted with an equal amount of salt solution and repeated every twelve hours until improvement occurs. The average total amount of serum required in the Hospital of the Rockefeller Institute was about 250 c.c. The injection of such large doses of serum is not entirely without possible harm to the patient because of reaction to the foreign protein. The possibility of severe serum sickness should further be taken into consideration. From evidence recently collected, more particularly in the United States Army, the value of anti-pneumococcus sera has been questioned. Parallel series of cases showed no important difference in mortality between series receiving anti-pneumococcus serum, normal horse serum or no serum whatever. The patients were young men who had passed rigid physical examinations and therefore were good risks in acute infections. It does not follow that other classes of patients would show the same results. There is also great variation in the mortality of different epidemics, and also normally in different ages, so that only a sufficiently large number of treated cases extensively controlled will form a trustworthy basis of actual comparison as to the death rate, which is, after all, the final criterion as to the actual value of the serum. Cecil and Blake have recently examined the question on the basis of experiments with monkeys. They find that the administration of normal horse serum has no beneficial effect on experimental pneumococcus Type I pneumonia but that the intravenous administration of specific Type I antiserum, particularly if given early and frequently, "exercises a specific therapeutic effect, frees the blood promptly and permanently from pneumococci, shortens the course of the disease and greatly moderates its severity." The treatment of lobar pneumonia with Cole's serum at present is best carried out in institutions where it is possible to make accurate bacteriological diagnosis and differentiation of the types of the cocci, and where intravenous administration of large doses of sera can be accomplished with the largest margin of safety to the patient.

Kyes has carried out extensive investigations on the clinical value of a serum produced by injecting massive doses of virulent pneumococci into the domestic fowl. The reason for the selection of the fowl as supply animal is that no matter how virulent pneumococci are for other species, they do not occasion disease in fowls, and therefore large doses can be injected with impunity. The initial dose in most instances is a surface growth equal to that of 240 test-tube slants. The average

subsequent doses are approximately 400 test-tube slants each. All the injections are made intraperitoneally. Injections are given every two weeks over periods of from four months to two years. One week after the sixth injection a trial bleeding is made and thereafter at intervals of two weeks, alternating with the biweekly injections. The sera possess a high content of agglutinins and bacteriolysins and also exhibit a marked therapeutic influence upon infected animals. Clinically the serum is used in doses of 2.5 c.c., and injections made slowly. A majority of the cases have received one injection daily, but not infrequently two injections are given the same day. The injections are continued until the temperature remains below 100° F. Of 538 cases not treated, 244 cases died, the death rate being 45.3 per cent. Of the 175 similar cases treated with serum the death rate was 20.8 per cent. In the ward in which the serum was employed the death rate during the six weeks prior to the introduction of the serum treatment was 55 per cent. During the six weeks subsequent to the withdrawal of the serum treatment, the death rate was 51 per cent. These results are distinctly encouraging. McClelland has recently reported the results in 322 cases of lobar pneumonia in soldiers at Camp Grant in which treatment with fowl serum was given and concludes that the low mortality (7.7 per cent.) together with the favorable modification of clinical symptoms by the serum would seem to indicate the extension of its use in pneumococcus pneumonia. Considering the fact that these cases were in selected young men of military age, and that the author does not give a comparative mortality among non-treated cases, much of the value of this paper is lost. The serum has also been used by Litchfield with great benefit in a series of pneumococcus meningitis cases. Gray employed the Kyes serum in 234 cases of pneumococcus pneumonia with a mortality of 16.8 per cent., whereas in similar cases treated in the same way except that they received no serum, the mortality was 63.6 per cent. Much laboratory and clinical work remains to be done before any definite conclusive evidence as to the value of polyvalent or antigroup sera can be drawn with any degree of safety. Pneumococcus sera act in part by opsonization of the cocci, thus favoring phagocytosis. The standardization requirements of the Hygiene Laboratory, Washington, call for a serum that shall protect white mice against Type I pneumococcus only. It is felt by Ferry and Blanchard and many others that a potent polyvalent serum is an absolute necessity. These authors recently succeeded in immunizing horses with Types I, II and III and some strains of Type IV. This serum in doses of 0.2 c.c. protected mice against infection of Types I, III and IV organisms (ten million M.L.D.).

Anti-cholera Sera.—While antisera against cholera have been produced by several investigators, the treatment of the disease with these sera has not given the best of results. Metchnikoff, Roux and others have prepared sera against the toxins of organisms cultivated in colodion sacs. McFadyen used ground organisms. Kraus used the toxin of the El Tor vibrio as antigen. This organism was obtained by

Gottschliech in 1905 from the intestinal contents of pilgrims who had died at El Tor from dysentery, and is not a true cholera vibrio but very closely related to it. Kraus recommended his antitoxin for the treatment of the cholera. Schurupoff treated one-and-a-half to two-day-old cultures of the vibrio with alkali and injected this toxic material into horses at six to ten-day intervals. Under Kolle's direction, Carrière and Tomarkin injected horses and goats with cholera cultures containing also the toxic derivatives and used the mixed sera of these animals. They found that these sera are more valuable against cholera peritonitis of guinea-pigs than any other animal.

Ketscher and Kernig used Kraus' serum in 119 severe and moderately severe cases with a death rate of 58 per cent. in those who received subcutaneous injections, and 50 per cent. when used intravenously, while among the non-injected cases the mortality was 63.4 per cent. Others have found among the serum-treated cases a mortality of 57.5 per cent. and among the control cases 84.3 per cent. This serum was administered by Jegunoff intravenously together with physiological salt solution, giving at first 140 c.c. of serum with 500 c.c. to 700 c.c. of physiological salt solution and subsequently a second injection of 80 to 120 c.c. of serum within seven and one-half to twenty-three hours after the primary injection. During the Russian epidemics in 1908 and 1909 it was shown that large doses of sera did not harm the patients. It was originally believed that large doses of sera lead to quick destruction of the vibrios with subsequent intoxication, but this has not proven to be the case. During these epidemics Salimbeni's and Kraus' sera did not give satisfactory results. Schurupoff's serum was considerably better, and the best results were obtained with the serum prepared according to Carrière and Tomarkin. This serum was given in doses of 50 c.c. to 100 c.c. diluted with salt solution subcutaneously and intravenously and resulted in quick improvement. Von Stühlern and Tuschinski treated 149 algid cases with fifty-six deaths; twenty-five moderate and thirteen early cases were treated with no deaths. From a total of 187 cases the mortality was 29.9 per cent. The serum should be applied as early as possible. Cholera antisera contain bacteriolytic, agglutinative, probably anti-endotoxic, complement-fixing antibodies, and also tropins. Because of the variety of sera used and the inconclusive reports given it is exceedingly difficult to reach a definite conclusion regarding the curative value of anti-cholera sera. It seems to us that Carrière and Tomarkin's serum is the most promising.

The Use of Anti-anthrax Serum.—The treatment of anthrax has consisted mainly in excision of the pustule, application of chemical or thermal cautery, and the injection of germicides as iodine, mercuric chlorid or phenol in the regions of the pustule, but all these methods are objectional because they are likely to produce scars and disfigurement. Excision may furthermore increase the danger of systemic infection. Sclavo, Deutsch, Sobernheim and others have produced immune sera by immunization of the sheep, horse and ass with attenuated culture of anthrax bacilli. From the work of Marchoux we know

that this serum possesses prophylactic and therapeutic properties in animals. Anti-anthrax serum has been used for several years, especially in Italy, France and England, with encouraging results. Scavo treated his cases without excision and in a series of 164 cases treated with specific serum this author reduced the mortality from 24 per cent. to 5.3 per cent. Scavo recommends 30 to 40 c.c. serum administered subcutaneously in doses of 10 c.c. on the first day and repeated if necessary on the next day. In severe infections 10 c.c. were given by the intravenous route. In severe cases it is advisable to give the injections in massive doses of 80 c.c. to 100 c.c. and preferably intravenously. Shera advises administration of 20 c.c. every twelve hours until pyrexia ceases. Regan recently injected the serum (10 c.c. to 15 c.c.) into the tissues surrounding the pustule and found that it possesses none of the disadvantages of the previous methods of local treatment, and has a very rapid and complete effect on the pustule, not only in arresting its further development but also in producing a subsidence of all local inflammatory symptoms. He advises also general treatment either intramuscularly or intravenously. Local treatment in order to effect a cure must anticipate the onset of an anthrax septicemia. In case the organisms have been demonstrated in the bloodstream the prognosis is usually grave. In this case 200 c.c. of serum is not excessive, and if necessary should be repeated until a negative blood culture is obtained. In intestinal anthrax large doses of serum should be given by the intravenous route. Penna and Beltrami and Penna, Cuenca and Kraus have obtained good results with normal beef serum. Their mortality was 6.2 per cent. in 372 cases, while the mortality previous to this period of treatment was about 10 per cent. These authors advise the use of 30 to 50 c.c. of normal beef serum administered subcutaneously. If no improvement occurs the injections should be repeated every twelve, twenty-four or thirty-six hours, but it seldom happens that a patient requires more than two or three injections. In severe cases intravenous administration is recommended. Similar favorable results were obtained by Solari and Langon, but Lignières has reported unfavorably upon the curative action of normal beef serum, stating that it is inferior to horse anti-anthrax serum. He calls attention to the prevalence of anthrax in cattle as evidence of the apparent lack of natural resistance to the disease. More recently Kolmer, Wanner and Koehler pointed out on the basis of their experiments that normal beef serum as secured from animals under ordinary conditions is but feebly protective or curative for anthrax and while its administration as described by Penna and his associates may favorably influence the pustule it is doubtful if the serum is sufficiently powerful to influence anthrax bacteremia. According to Kolmer, cases with sterile blood culture always recover. The potent factor of anti-anthrax serum appears to be a thermostable opsonin.

The Serum Treatment of Plague.—The therapeutic value of anti-plague serum is still a matter of dispute. Plague epidemics are exceedingly variable in character. Irregularity in the gravity of the disease

in different individuals is of common occurrence. A wide variety of antisera has been employed, but no attempt has been made to standardize the different sera. In many instances the cases treated were especially selected and moribund cases excluded. The result is that much of the existing statistical data is unreliable. Yersin, Calmette and Borrel were the first to show that the serum of an animal immunized to bacillus pestis has protective qualities and Yersin is credited with the production of anti-plague horse serum. This serum was prepared by immunization of horses first with dead and subsequently with living bacilli. Tavell's serum was prepared on the same principle, but Hata and also Kraus immunized their animals with dead bacilli alone and claim that these sera compare favorably with sera produced by the injection of living bacilli. The use of dead bacilli minimizes the danger of laboratory infections. Soon after the discovery of the nucleoproteins by Ferrannini, Galeotti and Lustig employed nucleoproteins from plague bacilli as antigen for the production of anti-plague serum. For this purpose the bacilli were broken down in 1 per cent. KOH solution and the nucleoproteins precipitated by the addition of acetic acid and then suspended in salt solution. Rowland also used a similar antigen, and others have employed a variety of extracts as antigens.

The serum at present most commonly used is obtained from horses after repeated intravenous injections of killed cultures sometimes followed by living organisms. Experimentally the sera show considerable strength in protecting animals against infection and exhibit specific bacteriolytic, bacteriotropic, agglutinative and antitoxic qualities. The antitoxic titer is usually very low. According to Kraus, Yersin's serum is not any better than the sera prepared with dead bacilli or nucleoproteins. Yersin used his serum in twenty-six cases during the epidemics of 1896 in Canton and Amoy, China, with a mortality of 7.6 per cent., while the mortality in cases not treated with serum reached 80 per cent. to 90 per cent. In 1897 141 cases were treated in Bombay and Cutch-Mandir with a mortality of 49 per cent. Of 685 cases not treated 80 per cent. died. In 1898 thirty-three cases were treated in Anam with Yersin's serum. The death rate among non-treated cases was 100 per cent. but was 42 per cent. among the serum-treated cases. It was found that the serum was entirely inefficient in cases with the pneumonic form of the disease. The German Commission at Bombay claimed that the low mortality (50 per cent.) of serum-treated cases was due to the selection of mild cases or cases arriving at the hospitals during the first or second day of their illness. Clemon also failed to obtain results in his fifty cases in which he injected as much as 60 c.c. of the Yersin serum. The Indian Plague Commission did not report favorably on Yersin's serum. Calmette and Salimbeni obtained very good results with serotherapy in Oporto, Portugal; of 142 treated cases twenty-one died, while of seventy-two not treated forty-six died. Kossel and Frosch and others studied this epidemic and found it to be of a mild type. During the Manchurian campaign serum treatments were entirely inefficient. Choksy injected large doses (100 c.c)

of the Parisian serum in his cases, repeating this six to eight hours later, and if necessary followed again by another injection. The next two days he administered 20 to 50 c.c., so that an adult received a total of 590 c.c. From a careful study he obtained a mortality of 72.5 per cent. among the serum-treated cases, and 82.3 per cent. among his control cases. This author also emphasizes the enormous advantage of early injections. In a series of 222 cases treated on the second, third, fourth, fifth, sixth and seventh day of illness he found the mortality as follows: 38.2, 56.7, 58.2, 50.8, 62.9, 60.0, and 75 per cent. respectively. According to Burnet, satisfactory results have been obtained in Queensland at the Colmolie Plague Hospital. Among 190 serum-treated cases during the period 1901 to 1907 the mortality was 29.7 per cent., while the mortality during the same period among non-treated cases was 73.9. Penna in Argentina injects massive doses 80 to 100 c.c. intravenously, and repeats the injection of 50 c.c. after twelve to twenty-four hours. Among 664 treated cases during the period 1905 to 1912 he reported a mortality as high as 23 per cent. in 1906, and a mortality of 7.3 per cent. in 1912, the average mortality was 12.5 per cent. From 1914 to the middle of 1919 Kraus' serum was used with an average mortality of 7.8 per cent. Kraus' serum, therefore, gave better results than Yersin's serum. Intravenous or intramuscular injections can be employed to ensure rapid absorption and the injections should be continued every twelve to twenty-four hours for two or more days until diarrhea has been controlled and the disease begins to subside. From all these studies we may conclude that although serum therapy of plague has not given striking results as diphtheria antitoxin in diphtheria, still it is the only specific means of combating the disease and when given early and in massive doses apparently influences the disease favorably.

Anti-bacterial Serum in the Treatment of Diphtheria Carriers.—Although Wassermann in 1902 recommended the use of a bactericidal serum, Martin was the first to use anti-bacterial serum in the treatment of diphtheria carriers. Martin injected diphtheria bacilli intravenously or intraperitoneally into horses and obtained sera with marked agglutinating properties. He claims that this serum has, when applied locally, the property of causing a rapid decrease in number of living bacilli in the throat. The best results were obtained by incorporating the dried serum with gum and using it in the form of pastilles. Dopter and many others have reported a decrease in the carrier period by the use of anti-bacterial serum. More recently Roskam and Arloing and Stevenin have called attention to the value of this method of treatment. Ecker immunized sheep with various strains of diphtheria bacilli, and by using massive doses obtained a potent agglutinative and lytic serum. To this serum fresh guinea-pig complement was added and the mixture sprayed by means of atomizers into the nasal passages, and over tonsils, fauces and pharynx four and five times a day. A total of forty-eight cases were treated, eighteen convalescent and thirty contact carriers. The duration of the carrier state after the introduction of the serum was seven

days, while the average duration of eighty-seven control cases was 18.6 days. A few cases proved to be persistent carriers. The less favorable results obtained by Kretschmer, Blumenau and Nolf may be explained by their small series of treated cases, weak sera and ineffective methods of application of the serum. Although the results so far obtained are not entirely convincing, the use of anti-bacterial serum in the treatment of diphtheria still deserves careful consideration. It is not possible to resort to tonsillectomy or adenoidectomy in all instances, and the majority of antiseptics are irritant. In many instances it is practically impossible to reach the organisms because they are buried in crypts, and tonsillectomy remains as the favored mode of treatment, although even this method is not invariably successful.

Anti-gonococcus Sera.—The early work of Rogers and Torrey has led to attempts at treatment of gonococcal infections by means of immune sera. Torrey's serum is prepared by injecting sheep with dead and subsequently living cultures of virulent strains of the gonococcus. Although efforts have been made to treat urethral, vulvar and vaginal gonorrhea by local applications of serum the disposition of the organisms in deep glands has been sufficient to result in the failure of this method. Recent studies of Debré and Paraf offer some encouragement for the treatment of gonorrheal rheumatism by the use of polyvalent sera, but they find that local injections about the site of the disease are more effective than general subcutaneous or intravenous injections. Further studies may demonstrate the value of serum treatment of chronic gonorrheal infections, but at the present time the method cannot be highly recommended.

Serum Treatment of Tuberculosis.—The best-known sera for use in tuberculosis are those of Maragliano and Marmorek. The first is prepared by immunizing horses with a mixture of a toxic filtrate of the bacilli and an aqueous extract of killed virulent tubercle bacilli. One cubic centimetre of the immune horse serum is injected into the patient every other day for a period of one and one-half months. A number of Italian workers found the serum effective, but other observers have not been convinced of its value. Marmorek's serum is prepared by inoculating horses with young tubercle bacilli poor in acid fast character. In addition, Marmorek immunized animals with pure cultures of streptococci obtained from the sputum of tuberculous patients. This serum is injected subcutaneously in daily doses of from 5 to 10 c.c. or intrarectally in doses of from 10 to 20 c.c. A number of workers, as for instance Wohlberg, have reported a favorable influence; others deny this effect. Wohlberg found the best results in scrofulous cases but not in cases of fully developed tuberculosis. The benefits of serum therapy of tuberculosis have not been convincing.

Serum Treatment of Typhoid Fever.—Lewin and Yes, Beumer and Pfeiffer and Chantemesse were among the first to produce antisera for this disease. Chantemesse's antiserum was prepared by immunizing horses with soluble toxins of the typhoid bacillus. Balthazard tested this serum and found it to agglutinate typhoid bacilli in very

high dilutions and to protect animals under experimental conditions. In 1000 cases of typhoid fever, Chantemesse reduced the mortality to 4.3 per cent., whereas the mortality among 5621 cases at the other hospitals in Paris not treated with serum was 17 per cent. Similar favorable reports were made by Brunon and Josias. Kraus and Stenitzer also produced antitoxic sera by immunizing their animals with soluble toxins and Cjaupp claims that the serum can be used with advantage in the treatment of the disease. Besredka and Lüdke prepared sera by immunizing horses and goats with the endotoxin of the typhoid bacillus, but it seems that the serum is not primarily an anti-endotoxin but rather a bactericidal serum which neutralizes both the exo- and endotoxins of the typhoid bacillus. According to Andriesen and Cinca, it can be used clinically. Sera were also prepared by immunizing animals with sensitized cultures of the typhoid bacillus and also with products obtained by digesting typhoid bacilli with trypsin. This toxic compound is known as "Fermotoxin" (Gottstein and Mathes). Rommel and Herman failed to obtain encouraging results with serum prepared by immunization with sensitized bacilli. The most favorable results, however, were secured by Rodet and Langrifoul. These authors immunized horses intravenously with both living cultures and old endotoxins, and in a summary of 400 cases Rodet finds that by repeated injections of this serum in doses from 10 to 20 c.c. given subcutaneously every other day the duration of the fever is markedly reduced in cases that are treated early. Serum treatment appears to reduce the bacteremia. It is also known that twenty-four hours after the injection of serum a definite increase in splenic dullness is observed, which presumably indicates a general stimulation of the lymphoid and myeloid tissue. The self-limitation of the disease, in the absence of complications, throws some doubt on the practical value of such sera.

AUTO-SERUM THERAPY

The use of the patient's own serum in the treatment of his disease has been suggested and applied by a number of workers. Gilbert, Marcon and many others treated tuberculous peritonitis and tuberculous pleurisy with effusion, by the subcutaneous injection of 1. c.c. to 2. c.c. of the patient's own serum and claim that the absorption of the exudate is greatly increased and an immediate improvement occurs. Eisner observed a leucocytosis following the injection of the serum in experimental tuberculous infections of rabbits and guinea-pigs and believes that this fact explains the favorable results reported in this method of treatment. Other investigators believe that specific antibodies favorably influence the process, but Levy, Valenzi and others are inclined to believe that the results are independent of the injections. It is possible that simultaneously with the transfer of the serum a minute amount of tuberculin is introduced. The exact nature of the phenomenon is, however, obscure. In influenza, Malta fever and typhoid fever Modinos has also obtained beneficial effects and Jez applied the treatment favorably in erysipelas. Capogrossi more re-

cently treated two cases of cerebrospinal meningitis with fairly good results. Hodenpyl treated a case of carcinoma with the patient's own ascitic fluid with apparent success. He used this fluid in large quantities in a number of cases but only with transient success. Risley also applied this method of treatment in sixty-five cases of cancer, using ascitic fluid from cancer cases and also other body fluids from non-cancerous cases. No encouragement for this method has been found in experimentally inoculated mouse cancers and the subsequent history of Hodenpyl's cases showed no permanent improvement. Auto-serum therapy has further been applied in obstinate and chronic skin troubles, such as psoriasis, dermatitis herpetiformis, pemphigus, lichen ruber, lichen planus, urticaria and squamous eczema. The serum is used in doses of 30 to 40 c.c. and repeated from two to six times at intervals of from three to five days.

Auto-serum Therapy in Syphilis.—Perhaps the most widely used auto-serum therapy is the salvarsanized auto-serum in the treatment of parasyphilis. The treatment of syphilis of the nervous system with salvarsan or neosalvarsan alone has not given the results expected. This is because the choroid plexus filters out these compounds, preventing their entry into the cerebrospinal fluid. It has been shown by Plaut that the serum of patients who have received salvarsan possesses antisyphilitic power, while normal serum fails to display this characteristic. Similarly Meirowsky and Hartmann and Gibbs and Calthrop obtained good results in the subcutaneous treatment of lues with serum of salvarsanized patients. According to Swift and Ellis, salvarsanized serum inhibits the treponema more intensively if heated to 56° C. for half an hour. These facts formed the underlying principles for the treatment of late syphilis with salvarsanized serum. Swift and Ellis injected salvarsanized serum intrathecally in a number of cases of tabes dorsalis and in other manifestations of neurosyphilis, and reported most encouraging results in both clinical and immunological manifestations. This work has since been confirmed by a large number of authors. The treatment is of special value in the earlier stages of neurosyphilis. Unfavorable results have been observed, as for instance the spasmodic retention of urine. As a result of long standing of the salvarsanized serum prior to its use, the drug may become oxidized with a marked increase in toxicity.

Method of Treatment.—Six-tenths to nine-tenths gram of salvarsan or neosalvarsan is injected intravenously. One hour later 40 c.c. of the patient's blood is withdrawn, allowed to coagulate and centrifuged. Twelve cubic centimetres of the sterile serum is diluted with 18 c.c. of sterile physiological salt solution to make it a 40 per cent. dilution and heated for half an hour at 56° C. A lumbar puncture is then performed, and 25 to 30 c.c. of fluid is withdrawn, and the serum very slowly injected. Swift and Ellis recommend the gravitation method of injection to prevent a sudden increase in intrathecal pressure. The patient is then kept in bed for twenty-four hours and the foot of the bed elevated for part of this time. The reaction is usually of a mild

type, including slight fever, pain in the legs, but in rare instances violent symptoms have been observed. Before and after the treatment a Wassermann test, the globulin test and a cell count should be made. After one week or more the treatment can be safely repeated until definite improvement occurs.

TREATMENT WITH IMMUNE HUMAN SERUM

Weisbecker in 1897 appeared to be the first to have used blood serum of convalescents, in cases of scarlet fever, but with little success. Huber and Blumenthal, von Leyden and others renewed the interest in convalescent serum therapy but failed to reach any definite conclusion probably because of the small doses employed. Reiss and Jungmann, Koch, Zingher and Weaver more recently applied the treatment with a fair degree of success. Reiss and Jungmann gave intravenous injections of 40 c.c. to 100 c.c. and drew the blood from scarlet-fever convalescents about the end of the third or beginning of the fourth week of the disease, testing each serum for the possibility of syphilis and for sterility. Zingher injected citrated whole blood intramuscularly in doses of 120 c.c. to 240 c.c. and repeated in two or three days if necessary. Weaver drew the blood from convalescents between the twentieth to twenty-eighth day, only such convalescents being selected who had not been septic and who gave a negative Wassermann reaction. The sera were tested for sterility and used pooled. Intramuscular injections were given in doses of 25 c.c. to 90 c.c., 60 c.c. being the usual amount. The effects of the serum are rapid and start with a sudden drop in temperature and general improvement of the patient within twenty-four hours after the administration of the serum. The best results are obtained when the patients are treated early in the disease. Kling and Widfeldt also reported favorable results in their series of cases during an epidemic of 237 cases at Stockholm in 1918. This method has not been widely adopted and there is still much question as to whether improvement is due to the treatment or to the natural self-limitation of the disease.

Monvoisin has recently reported encouraging results in typhus fever by intravenous injections of human convalescent serum. One or two cubic centimetres of serum brought a marked drop in temperature and general improvement in the patient. Monvoisin noted a decrease in mortality from 30 down to 10.34 per cent. by the use of convalescent sera. The serum was obtained from a patient on the eighth day after subsidence of fever. Favorable results were also reported by Teissier in cases of severe and hemorrhagic smallpox. In leprosy the serum obtained from cantharides blisters on lepers has been reported to be of value.

Bleyer recently injected immune human blood into a series of forty-five cases of whooping-cough in the early weeks of the disease. This series was divided into three groups. The first group received blood from persons who were convalescent or who had recovered from whooping-cough within three months. In the second series the blood

was from persons who had the disease at more remote periods, and the third group from persons who, so far as they knew, had never suffered with whooping-cough. The stage at which the treatment was given was about the same in the three groups and the dosage depended upon the body weight of the patient, varying between 40 c.c. and 125 c.c., divided into two, three or four doses and injected into the gluteus muscles. In the first group there were no deaths and no complications, and the course of the disease was in no definite way different from the usual course. The second group showed quite as satisfactory improvement as in the first group. In the third group there were two pneumonia cases with one death and one case which apparently was favorably influenced by normal serum treatment. The groups are so small and the difference so slight as to give no reason for regarding this mode of treatment as particularly effective. Vaccine treatment of this disease gives much greater promise of success.

During the recent great epidemics of so-called influenza, convalescent serum was used in a considerable number of cases which developed pneumonia. In many instances there was marked improvement, but there is no clear indication that the results were specific or that they depended absolutely upon the serum treatment.

SERUM THERAPY IN INFECTIONS OF UNDETERMINED ETIOLOGY

Introduction.—The preparation of the immune sera discussed above depends not only upon knowledge of the etiological agent of the disease concerned but also necessitates the isolation of the organism in pure culture. Several infectious agents are known to exist in blood and tissues, since the diseases may be transmitted by means of inoculation of blood, organs or organ extracts. Many of these agents are so small as to pass through porcelain filters and are spoken of as the filterable viruses. Some of these viruses have been observed to contain minute globoid bodies which have been obtained in pure culture, but under such conditions that they have not served well as antigens for the production of immune sera. If immunization be attempted by injections of the blood or tissues containing the infective agents, the resulting immune serum contains not only antibodies for the infective agent but also for the tissues. If these tissues happen to be from the same species into which the serum is to be injected the hemagglutinins, hemolysins and cytolsins in the immune serum may seriously damage or even kill the individual so treated. Active immunization by the use of infected tissues appears to progress favorably in spite of the presence of the tissues, as seen in the active immunization of man and other animals by the use of the virus of rabies contained in the dried spinal cords of rabbits. It is in the production of sera for passive immunization that the danger from simultaneously formed tissue antibodies appears. Rous, Robertson and Oliver have studied this problem with a view to removing from the immune serum these harmful elements. After the immune serum is prepared the tissue antibodies are removed by selective absorption with red blood-corpuscles, since these cells re-

move the most important source of danger, the hemagglutinins and the hemolysins; undoubtedly many of the other tissue antibodies, as the cytolsins, are reduced in amount. For example, they immunized a goat with megatheriolysin and finely-ground liver, spleen and kidney, as well as defibrinated blood, of guinea-pigs. The immune serum was then repeatedly mixed with guinea-pig blood-cells until all the hemagglutinin and hemolysin had been removed. The process did not reduce the titer of the special antilysin against megatheriolysin either in test tube or animal experiments. Guinea-pigs were protected against megatheriolysin by the use of this serum and the treatment of the serum by selective absorption removed practically all the elements dangerous for the guinea-pig. Similar experiments were performed using as antigen the blood of rabbits suffering from pneumococcus septicemia. It was found that absorption, by means of blood, of anti-poliomyelitis serum produced no change in its protective value. Experiments were also performed with the Rous chicken sarcoma, a tumor caused by a filterable virus. The immune serum was prepared by injecting into geese a mixture of tumor tissue and the blood of moribund fowl since under these circumstances the blood contains the causative agent. The immune serum was treated with fowl blood-corpuscles to remove the tissue antibodies. The serum so treated, when employed in proper ratio to the amount of tumor inoculated, served to protect fowl against the subsequent growth and development of the tumor, whereas growth proceeded regularly in the unprotected controls. Rous makes no claim as to high protective value but that some such power is developed is undoubted.

The work quoted above is of the utmost importance in establishing the important principles that must be observed in the preparation of immune sera against infective agents either known or unknown when used as antigens in animal tissues. The studies are recent and have not as yet been widely applied. The immune sera against infections of undetermined cause to be described in this section were studied before the work of Rous and his associates appeared, and it is probable that the methods of preparation may be considerably modified in the course of time. The inclusion of acute anterior poliomyelitis in this group is justified only on the ground of dissension as to whether the disease is due to the globoid bodies described by Flexner and his collaborators or to the pleomorphic streptococcus studied by Rosenow, Nuzum and others.

Anti-poliomyelitis Serum.—That one attack of poliomyelitis protects against subsequent infection has been known for many years. Levaditi and Landsteiner and also Flexner and Lewis in 1910 demonstrated that the serum of convalescents and of monkeys recovered from the disease protects against infection. Treatment of human cases of the disease was applied by Netter in 1916. This author injected intrathecally the serum of recovered patients in doses of 5 to 13 c.c. for a period of eight days with most encouraging results. He believed that the best serum is found in individuals whose acute attack

dates back from three months to four years. Flexner carried out experiments with monkeys and proved that the serum of recovered cases was efficacious in the cure of these animals. In 1916-1917 this author used the serum extensively during the epidemic in New York and recommends the combination of intraspinal and intravenous injections. Children were given combined doses of 5 to 10 c.c. intraspinally and 30 to 40 c.c. intravenously. The possibility of conveying the disease is not considered a danger, because the virus has never been detected in the blood. The only difficulty encountered in this method of treatment is that of securing sufficient quantities of serum. Pooling of sera is of the greatest advantage, since the antibody content may vary widely in the sera of different persons.

During the epidemic of 1917 Mathers, Rosenow, Towne and Wheeler, Nuzum and Herzog, and later Nuzum reported the discovery of a pleomorphic streptococcus which they had constantly observed in the brain and spinal cord, and also in the cerebrospinal fluid in human cases of poliomyelitis. Flexner and Noguchi, Smillie and many others deny the etiological importance of this streptococcus. Rosenow, Nuzum and Willy claim to have produced sera with definite protective and curative effects. In the hands of Nuzum and Willy serum treatment reduced the mortality in a series of 159 cases from 38 per cent. to 11.9 per cent.

Amoss reported that only imperfect success in developing antibodies in rabbits and monkeys has attended the repeated injection of cultures of the globoid bodies of Flexner and Noguchi and also failed to find evidence that Rosenow's serum is either therapeutically effective in monkeys or possesses antibodies of the same nature as those present in the blood of monkeys which have recovered from experimental poliomyelitis. Since the antibodies in convalescent poliomyelitis serum in man and monkey are identical, this author states that any antibodies present in Rosenow's horse serum do not conform to those occurring in human convalescent serum. Again Amoss and Eberson in a later paper concluded that the anti-streptococcus serum of Nuzum and Willy fails to show in the monkey neutralizing or therapeutic power against small doses of the virus of poliomyelitis. Under the same conditions the serum of monkeys which had recovered from experimental poliomyelitis proved neutralizing and protective. These facts leave some doubt as to the actual value of anti-poliomyelitis horse serum, and until more conclusive evidence has been brought forward by the supporters of the streptococcus as an etiological factor we believe that the only effective serum existing is that of convalescent or recovered cases. Neustadter and Banzhaf immunized horses against a filtrate obtained from the digested brain and cord of a human case of the disease. The immune serum gave encouraging results in a few experiments with monkeys, but as yet data are too limited to justify a conclusion as to the usefulness of this serum.

Rinderpest.—Kolle and Turner injected gradually increasing doses of virulent rinderpest blood and also bile of infected animals into oxen

and obtained potent sera against the rinderpest virus. Of 3318 animals treated with this serum 455 or 13.9 per cent. died, while the mortality among non-treated animals averages between 85 per cent. and 95 per cent. The serum can be used prophylactically in doses of 100 to 200 c.c. If the virus is simultaneously injected in small doses as advised by these authors, the results appear to be extremely satisfactory. The serum for curative purposes should be employed within thirty days after the onset of fever.

Anti-hog-cholera Serum.—Immunization against hog cholera has an important historical as well as a practical bearing since it was in this disease that the first attempt to immunize with bacterial products was made. Salmon and Theobald Smith published in 1884 their account of the production of immune sera in the pigeon by the inoculation of killed broth culture of the bacillus of hog cholera. Subsequent studies have made it appear that the disease is not due to the bacillus of hog cholera and much evidence is at hand to support the view that the etiological agent is a filterable virus. At the present time immunity is produced in healthy hogs by the injection of blood obtained from infected hogs, thus implanting the virus. It is necessary to protect the animals employed by passive immunization with a previously-prepared antiserum. The animals selected are injected subcutaneously with 40 c.c. of anti-hog-cholera serum per hundred pounds of weight. Two to three days later the animals receive intravenously 3 or 4 c.c. of defibrinated blood obtained from an animal suffering from the disease, or the animals may be exposed in infected pens. If the animals survive, after a period of one month they are given 5 c.c. of the living virus. This is repeated after two or three weeks. The immunized animals are bled from the tail. Five cubic centimeters of blood per pound of weight are usually withdrawn. The protective power of the serum thus obtained is then determined in a series of hogs. For prophylactic purposes the animals receive 40 c.c. subcutaneously per hundred pounds of weight or simultaneous injections of virus and serum, but this combination is not without danger. For therapeutic purposes several injections are necessary and the serum should be administered as early as possible.

THERAPEUTIC USE OF NORMAL SERUM

Normal serum therapy in man has included the use of both human and animal sera. In the treatment of natural or experimental disease in man or animals the normal serum employed may be homologous or heterologous. The basis of such method of treatment has often been entirely empirical, but as serum therapy has been more carefully studied the employment of normal serum may be placed in two categories, namely that of the non-specific protein treatment of disease or that of providing the blood with certain elements necessary for the process of clotting. It is to be conceded that a normal serum may be employed because of some natural antibodies which it may contain, but such a form of passive immunization is much improved if the

natural antibodies are increased by specific immunization. The use of normal serum in non-specific therapy probably increases those non-specific factors of defense such as fever, serum enzymes, etc., that have already been discussed. In hemophilia, purpura hemorrhagica, melena neonatorum and similar hemorrhagic diseases there is a disturbance of proper balance of those constituents of the blood and tissues which provide for coagulation of the blood. Hypotheses differ as to the exact mechanism of the process of coagulation, but fundamentally it seems necessary to have an equilibrium of prothrombin and antithrombin. This balance may be upset by an excess of antithrombin, by a deficiency in prothrombin, fibrinogen, calcium salts or other elements. The interaction of prothrombin, thrombokinase (or thromboplastin) and calcium salts results in the formation of thrombin. Thrombin and fibrinogen interact to form fibrin, the essential element of a clot. Blood serum is rich in prothrombin and if a hemorrhagic disease be due to prothrombin deficiency, serum treatment is likely to be beneficial. If, on the other hand, the disease be due to an excess of antithrombin the introduction of prothrombin has little value. Similarly hemorrhagic disease with low fibrinogen content is not benefited by serum treatment. Whipple has found decrease of fibrinogen in advanced cirrhosis of the liver with hemorrhage, excess of antithrombin in aplastic anemia and leucemia and deficiency of prothrombin in melena neonatorum. Duke holds that the lack of prothrombin is due to a deficiency in the number of platelets, whereas Minot and Lee believe that in hemophilia, at least, the slow clotting is due to a hereditary defect in the platelets which renders them less available for the process of coagulation. Various studies have given different results as to the changes found in the elements concerned in clotting. Whipple points out that if the phenomenon is studied in the individual case rational therapy may be applied. In melena neonatorum the administration of blood serum often gives brilliant results. In other hemorrhagic diseases the results are somewhat more variable. If hemorrhage has been severe and anemia is marked, the double purpose of favoring clotting and replacing lost blood may be served by transfusion from a suitable and properly-tested donor. The more direct the transfusion the less likelihood is there of alteration of the blood due to beginning clotting and the greater is the probability of contributing substances to replace or augment those which may be deficient in the patient's blood.

APPENDIX B

PROPHYLACTIC VACCINATION

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SENSITIZED VACCINES.

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PREPARATION OF BACTERIAL VACCINES.

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TYPHOID AND PARATYPHOID FEVERS.

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CHOLERA.

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OTHER DISEASES.

VACCINATION

Introduction.—In contrast to the methods of passive immunization, *i.e.*, the parenteral introduction of immune sera, vaccine treatment aims to increase the resistance to disease by the injection of the causal organisms or their products. The duration of this increased resistance varies in time according to species and types of organisms injected and the individual characteristics of the subject. For instance, vaccination

against smallpox immunization may last for a considerable number of years, while with other organisms, such as the staphylococcus or pneumococcus the immunity is of relatively short duration.

The aims of vaccination are either to cause prophylactic resistance against disease or to increase an already established resistance. Prophylactic vaccination against typhoid is an example of the former, while the vaccine treatment of furunculosis or gonorrhea are examples of the latter.

The term vaccine is derived from vaccinia or cowpox, and the method of protective immunization against smallpox with vaccinia virus was called by Jenner "vaccination." This great empirical work was placed on a sound scientific basis by Pasteur after he had discovered the method of protective inoculation against chicken cholera, and Pasteur used the term vaccination for such inoculations. To-day the simple term vaccine is loosely applied and should be restricted to cowpox vaccine. Suspensions of bacteria such as typhoid bacilli or pyogenic cocci should be designated bacterial vaccines. Wright defines a bacterial vaccine as follows: "Bacterial vaccines are sterilized and enumerated suspensions of bacteria which furnish, when they dissolve in the body, substances which stimulate the healthy tissues to the production of specific bacteriotropic substances (or antibodies) which fasten upon and directly or indirectly contribute to the destruction of the corresponding bacteria."

Perhaps the first serious attempt to apply practically a bacterial vaccine in the treatment of human disease was that of Koch, who in 1890 employed tuberculin in the treatment of tuberculosis. In 1893 Fränkel treated thirty-seven cases of typhoid fever with subcutaneous injections of killed typhoid bacilli. He reported that the course of the disease was favorably modified and in a few instances terminated by rapid lysis. Rumpf treated a series of cases of typhoid fever with bacillus pyocyaneus and obtained equally favorable results, thus throwing doubt upon the specific character of the treatment and leading into the newer field of non-specific therapy. Wright and Douglas soon after the discovery of opsonins demonstrated their method of treatment by bacterial vaccines under the guidance of the opsonic index. Wright stated that a patient who had become infected by an organism such as the staphylococcus aureus or the tubercle bacillus would be found to have a lowered resistance against these organisms; that this degree of want of resistance could be accurately determined, and that the resistance could be stimulated and controlled by measured doses of a vaccine of the causative organism. Wright's method of treatment was based on the principle of strict specificity. It was soon pointed out that opsonins are only one link in the defensive chain of the host, and the use of the method has been somewhat restricted. The measure of opsonins in a given instance was subsequently found not to be a measure of the existing degree of total immunity. In the majority of diseases, therapeutic vaccination has not withstood the test of time. Wright himself, after experiences in the World War, stated that it has been

accepted that the inoculation of microbes into the already infected system is as illogical as to instil further poison into an already poisoned body. However, a wide field for prophylactic vaccination is still open. Soon after Wright's work bacterial vaccines were applied in every conceivable way and unfortunately much harm has been done to the rational use of vaccines by reckless commercialism.

Wright and his collaborators have studied carefully the opsonic index of patients the victims of infectious disease as well as that of normal individuals. They found that phagocytosis is often depressed in those who are unsuccessfully combating certain disease and that the phagocytic power can be increased by specific bacterial vaccination. They pointed out further that following the first dose of vaccine the opsonic index is considerably depressed and spoke of this phenomenon as the negative phase. This phase may last for several days and numerous writers have thought that such a depression of phagocytic resistance might indicate such a decrease of general immunity as to render vaccination during an epidemic highly undesirable. The negative phase has been carefully investigated and many now believe that it does not exist. The factor of error in the determination of the opsonic index is considerable, owing to the variability of conditions operating *in vitro*. Therefore, it is possible that the decrease of index pointed to by Wright may fall within the limit of experimental error. The recent observations of Balteano and Lupu indicate that no such negative phase is demonstrable in cholera, and the careful investigations of Cantacuzène indicate that the negative phase does not occur in other diseases.

Types of Vaccines.—Living Vaccines.—From animal experiments it is generally admitted that the greatest and most lasting immunity is produced by the injection of living bacteria. The killing of bacteria apparently destroys certain thermolabile substances which possess antigenic properties. In human practice the use of living bacteria is not without danger. One may at first inoculate with a single living organism and cautiously increase the number, but the virulence of the organism is not easily controlled and may be so great as to make such inoculations dangerous. In addition there is a risk of establishing a "carrier state" since the gradual increase of the number of organisms may establish a mutual immunity on the part of both the parasite and the host. If the virus of the disease can be so attenuated that danger of producing an outspoken attack of the disease is eliminated, vaccination can be performed with great success. The outstanding examples of this method in human medicine are vaccination against smallpox and against rabies. In smallpox the virus is attenuated by animal passage through the calf and in rabies the virus is attenuated by desiccation.

Sensitized Vaccines.—These are bacterial vaccines composed of bacteria which have been exposed to their specific immune serum. As early as 1891 Babes mixed the blood of a highly refractory dog with an emulsion of street virus in order to produce in other animals a more

rapid development of immunity against rabies. In the only experiment reported at this time it was shown that some protection was afforded by the mixture, although the inoculated animal finally succumbed to rabies. Lorenz in 1892 made similar observations in swine erysipelas. Since this time numerous workers have used the method. The most important advance was made when Besredka suggested the removal of the excess of serum by centrifugally washing the sensitized bacteria. Subsequent work has been carried on with killed bacteria treated with their immune sera, washed and suspended in a suitable menstruum. The ordinary non-sensitized bacterial vaccines injected into an animal during the incubation period of a disease are likely to hasten the death of the animal, or if the infection is already acquired, the injection of the vaccine appears to lower the natural resistance. Besredka and Metchnikoff believe that sensitized bacterial vaccines produce no negative phase, but only slight local and general reactions and facilitate the production of antibodies. Kakechi has shown that the toxicity of sensitized bacterial vaccines is less than that of the non-sensitized. Sensitized bacterial vaccines have been employed in numerous infectious diseases such as typhoid fever, asiatic cholera and bubonic plague with varying degrees of success.

Killed Bacterial Vaccines.—These are suspensions of bacteria usually in salt solution but sometimes in other menstrua such as neutral oil. The organisms are usually killed after the suspension has been made, but in making oil suspensions the organisms are killed before the final suspension. Heat is usually employed for killing the bacteria and the action is further supplemented by the addition of a bactericidal preservative to the suspension. Under certain circumstances chemicals such as formaldehyde or phenol may be employed both for killing and preserving the vaccine. *Autogenous vaccines* are bacterial vaccines prepared from bacteria which have been freshly isolated from the individual patient. At times it is very difficult to isolate the organism as for instance in gonorrhea. In these cases stock vaccines are usually employed. *Stock vaccines* are made from strains of bacteria isolated at some previous time and kept in the laboratory stock. Stock vaccines are used extensively in prophylactic vaccinations. *Mixed vaccines* are composed of various kinds of bacteria. Their value is questionable and their use unscientific, except on the basis of non-specific therapy. Many efforts have been made to produce the bacterial antigen in a pure form so as to obtain a minimum of local and general reaction, and to immunize in the shortest space of time possible. Such vaccines have been made from nucleoproteins, autolyzed bacteria, digested bacteria and detoxicated organisms. It appears that some of these methods are promising, especially for the production of antigens from spore-bearing bacteria.

Preparation of a Bacterial Vaccine.—Under strict asepsis an emulsion of the organism in question is prepared by adding 5 to 10 c.c. of physiological salt solution to a twenty-four-hour agar slant culture. This is allowed to stand ten minutes and then rotated actively in order to make a suspension of the organisms. The suspension is now filtered through sterilized filter paper in a funnel into a

sterile test-tube. In case of scanty growth the emulsion is directly transferred to another surface culture, the growth in this tube suspended and the process repeated with additional growths until a satisfactory emulsion is obtained. Instead of filtering the emulsion one may shake the emulsion in a test tube containing glass beads to break up the clumps. It is of great importance to have a homogeneous suspension. Because of the presence of pepton or proteins from the culture media some authors advise washing of the organisms until the supernatant fluid gives a negative biuret reaction. The next step in the preparation is the counting of the emulsion. This can be done by the hemocytometer method, by Wright's method and other methods.

Hemocytometer Method.—(From Zinsser, Hopkins and Ottenberg, "A Laboratory Course in Serum Study.")—A staining solution is prepared by adding to 20 c.c. of 1 per cent. phenol 1 c.c. of a saturated alcoholic solution of thionin. A small amount of the carefully shaken bacterial suspension is removed to a watch glass. A dilution of 1-100 is prepared in a red cell pipette with the staining solution as diluent to the 101 mark. After carefully shaking and after blowing out the portion of the fluid in the capillary end of the pipette a small drop is placed in a counting chamber and covered with a flat coverslip. After allowing fifteen minutes for the bacteria to settle a count is made, with 4. mm. objective, of a number of squares until 200 or more bacteria have been counted. It is best to take this count from different portions of the ruled surface and from two separate drops of the mixture. The small squares have an area of 1/400 of a square mm., the depth of the chamber is 0.1 mm., the dilution is 1-100. The number of bacteria may be estimated by the following formula:

$$\frac{\text{Number of bacteria counted} \times 400 \times 10 \times 100 \times 1000}{\text{Number of squares counted}} = \text{number of bacteria in 1.0 c.c.}$$

Wright's Method.—A drawn-out capillary pipette is prepared and marked with a grease pencil about 2. cm. from the tip. A small puncture is made in the tip of the finger and a fresh drop of blood obtained. Three units of salt solution are then drawn up in the pipette, admitting a bubble of air between each unit of salt solution. The unit is the amount that is drawn up to the mark on the pipette. Blood from the finger-tip is then drawn up to the mark, a bubble of air admitted and the bacterial suspension drawn up to the mark. The mixture is then blown out on a clean slide and drawn in and out of the pipette several times to ensure even mixing of the blood and bacteria. A drop of this mixture is placed on a second slide and carefully spread across the slide in the manner of making blood smears. It is important that the film be thin and even, so that the red cells are not piled in masses in any portion of the film. This film is stained with Wright's stain, or by any other simple method, and a differential count of the number of bacteria and red cells in a number of fields in different parts of the slide is made. For this a rule scale to be inserted in the eyepiece of the microscope is very helpful. Fields are counted until 200 red cells have been counted. The number of bacteria in the suspension may then be estimated from the number of bacteria counted, using the following formula (assuming that the blood of the worker contains 5,000,000 red cells per cmm.):

$$\frac{\text{Number of bacteria} \times 5,000,000 \times 1,000}{\text{Number of red cells (200)}} = \text{Number of bacteria per c.c.}$$

Other Methods.—Among the other methods of standardization of the suspension are the comparison of the emulsion with a known standard emulsion, the estimate of the average number of organisms per slope grown in, say, eighteen hours, or an estimate of the number of germs per loopful (Kolle's method). Hopkins centrifugalized his suspension at high speed in a special tube with graduated tip until the supernatant fluid was clear. The number of organisms for a number of species in such a closely-packed sediment has been determined and is as follows:

Staphylococcus aureus	0.01 c.c. equals	10 billion
Streptococcus hemolyticus	0.01 c.c. equals	8 billion
Gonococcus	0.01 c.c. equals	8 billion
Pneumococcus (capsulated)	0.01 c.c. equals	2.5 billion
B. typhosus	0.01 c.c. equals	8 billion
B. coli	0.01 c.c. equals	4 billion

Gates recently standardized his bacterial suspension by measuring the opacity of the suspension by the length of the column of the suspension required to cause the disappearance of a wire loop. By a simple formula the measured opacity is translated into terms of the concentration of bacteria per cubic centimeter and so made comparable with that of other suspensions of the same organism.

The stock suspension after estimation of the number of organisms contained is ready for dilution. Shera employs the following method for dilution. Suppose the suspension is found to contain 6400 million organisms per cubic centimeter, and that a vaccine of 1000 millions per cubic centimeters is required. Five cubic centimeters are measured out accurately after shaking well, and they are made up to 6400/1000 parts, i.e., 6.4 parts. Multiply 6.4×5 and the result 32 equals the volume in cubic centimeters to which the 5 c.c. should be made up. The suspension is sterilized by means of heat. For staphylococci and streptococci 50° to 60° C. for half an hour is sufficient; for typhoid bacilli 50° to 56° C. for an hour is usually employed. It is best to add some preservative as phenol or tricresol (0.3 to 0.5 per cent.) to the suspension and to have the suspension in sealed ampoules preferably of brown glass before immersing in the water bath. Connor successfully sterilized his staphylococcic vaccines by means of fluorides. After sterilization an ampoule should be opened so that a culture may be made. No vaccine should be used until a culture is found to show no growth. If ampoules are not at hand they may be made from test tubes or the vaccine may be kept in sterile bottles with rubber stoppers or caps.

Dosage of Organisms.—For gonococci, bacillus coli, streptococci and pneumococci 5,000,000 to 50,000,000 are usually employed, while for staphylococci 200,000,000 to 1,000,000,000. Wilson gives the following minimum and maximum doses: Streptococcus, 6 and 68 millions; staphylococcus, 150 and 900 millions; gonococcus, 45 and 900 millions; meningococcus, 300 and 900 millions; micrococcus melitensis, 700 and 1400 millions; bacillus coli, 16 and 240 millions; bacillus typhosus for treatment, 100 and 250 millions; bacillus typhosus for prophylaxis, 500 and 1000 millions; bacillus pyocyaneus, 34 and 1000 millions; bacillus pneumoniae, 44 millions; bacillus tuberculosis, 1/2000 to 1/200 mg.

Lipovaccines.—Recently LeMoignic and Sézary showed that it is possible to obtain as highly hemolytic serum by injecting red cells suspended in oil as by injecting them suspended in salt solution. They also showed that the oil suspension gives slow absorption, and that the oil acts as a detoxifying agent. As an example of the rate of absorption it was shown that the injection 0.35 mgm. of strychnine in aqueous solution kills a guinea-pig, but the injection of six times that amount is harmless if the strychnine be dissolved in oil. This led LeMoignic and Pinoy, Achard and Foix, and LeMoignic and Sézary to suspend bacterial vaccines in oil, and to inject the entire vaccinating dose at one time. Bacterial vaccines suspended in saline are rapidly autolyzed. As autolysis advances, absorption following injection becomes more rapid and the immediate reaction more severe. Oil vaccines are preserved much more easily than saline vaccines and the reactions following their injection are less severe. The oil vaccines are known as "lipovaccines." The bacteria have been suspended in lanolin, lecithin, sperm oil and many vegetable oils. Cotton-seed oil is at present widely used. It is of great importance to use neutral oils. The sterilization of these oils has been a difficult problem and a drawback in the preparation of the vaccines. The technic must be strictly aseptic. At present lanolin and oils are sterilized in the autoclave at fifteen pounds for

fifteen minutes. Ultra-violet rays have been used. Chlorine has also been employed, but the resulting hydrochloric acid is difficult to remove. Whitmore and Fennel used powdered potassium iodid. This was added to olive oil and sweet almond oil; iodine was liberated in sufficient amount to sterilize the oil, and was taken up in the oil molecule so that no free iodine could be detected. Sweet almond oil is sterilized in about three days, but it requires about ten days to sterilize olive oil. It is not known, however, how the suspension in oil affects the antigenic power of the vaccine, but certain workers claim to get better results than by the use of saline vaccines. Against the use of lipovaccines is the possibility of fat embolism from accidental entrance of the vaccine into a vein, but Graham considers this factor of minor importance since the amount of oil is small. He injected as much as 0.8 c.c. of oil into the ear vein of a rabbit and observed only a slight passing dyspnea and no other evidence of discomfort. Care should be taken, however, to administer the vaccine subcutaneously and to avoid veins. Drawing out the plunger of the syringe after the needle has been introduced determines whether or not an important vein has been entered. The upper arm beneath the insertion of deltoid muscle is usually selected for the injection. The region of the scapular or pectoral muscles may do as well.

Contraindications.—In prophylactic immunization it is of importance to ascertain whether the patient has latent or active infection. In active tuberculosis vaccination is considered dangerous. Caution should be observed in diabetes, parenchymatous nephritis and carcinoma.

VACCINATION WITH LIVING VIRUS

Smallpox Vaccination.—Although inoculation with the virus in smallpox in an attempt to produce a mild attack of the disease had been practiced for centuries and although for many years it had been observed that an attack of cowpox rendered man immune to smallpox, it remained for Jenner in 1796 to furnish the scientific proof of the efficacy of vaccination with cowpox in the prevention of smallpox. Jenner's publications were so convincing that the method soon attained widespread use and was introduced into America in 1800 by Dr. Benjamin Waterhouse, of Boston. The work of the latter investigator was especially well conducted and convincing. In 1894 Copeman demonstrated the protection of monkeys against smallpox by vaccination with cowpox, and this was subsequently confirmed by Brinckerhoff and Tyzzer. The introduction of vaccination following Jenner's publication immediately led to marked reduction in the incidence of this disease and its mortality. The table on page 279 (taken from O'Connell, "Vaccination; What It Is, etc.," circular New York State Department of Health, 1908) gives a clear indication of the reduction of mortality.

Up until fairly recent times vaccination was practiced by inoculating patients with the fragments of the crust obtained from others who had been successfully vaccinated. This method has been aban-

done because of the possibility of transferring infection in the crusts, not the least important of which is syphilis. With the development of important Board of Health laboratories and large commercial laboratories it is now possible to secure cowpox virus in a form free from infective organisms.

DEATH-RATE FROM SMALLPOX, AVERAGE PER 1,000,000 INHABITANTS.

Before vaccination	After vaccination	Territory	Smallpox death rate, per 1,000,000 pop.	
			Before vaccination	After vaccination
1777-1806	1807-1850	Austria (lower)	2,484	340
1777-1806	1807-1850	Austria (upper and Salzburg)	1,421	501
1777-1806	1807-1850	Trieste	14,046	182
1777-1806	1807-1850	Tyrol and Vorarlberg	911	170
1777-1806	1807-1850	Bohemia	2,174	215
1777-1806	1807-1850	Moravia	5,402	255
1777-1806	1807-1850	Silesia (Austrian)	5,812	198
1776-1780	1810-1850	Prussia (East Province)	3,321	556
1780	1810-1850	Prussia (West Province)	2,272	356
1776-1780	1816-1850	Westphalia	2,643	114
1776-1780	1816-1850	Rhenish Provinces	908	90
1781-1805	1810-1850	Berlin	3,422	176
1774-1801	1810-1850	Sweden	2,050	158
1751-1800	1801-1850	Copenhagen	3,128	286

The Preparation of Smallpox Vaccine.—For this purpose cowpox is produced in young heifers from two to four months old. The animals are taken from selected stock, carefully tested for the presence of tuberculosis and observed for several days so as to ensure perfect health. The body is cleansed and the abdominal surface shaved from the ensiform cartilage to the pubis, extending the area out on the flanks and the inner surface of the thighs. The skin is washed with soap and water, then with alcohol and finally with sterile water. About 100 small cuts through the epidermis are made under strictly aseptic precautions. If bleeding occurs the blood is carefully wiped away. Virus may be obtained primarily from smallpox patients who are otherwise healthy. At the present time, however, the virus kept as "seed virus" is obtained from previously inoculated animals. Virus is introduced into the scarifications usually in the form of a glycerol suspension. In about forty-eight hours a reaction appears and by the sixth day the vesicles are well filled with semipurulent material. The animal is killed and the vesicles carefully curetted away. After the curettage, serum appears and this may be preserved in ampoules or small tubes for subsequent vaccination. The pulpy mass obtained by curettage is mixed with four times its weight of a mixture composed of glycerol 50 per cent., water 49 per cent., phenol 1 per cent. The glycerolated pulp is allowed to stand three or four weeks in order to destroy any contaminating bacteria. The pulp is then triturated in

special machines and sealed in capillary tubes. Formerly "ivory" vaccine points were also charged from this pulp, but these have been forbidden in interstate commerce (page 282). In all cases the material before being prepared for distribution is carefully tested for the presence of tetanus bacilli or their spores. Its potency may be determined by directly inoculating the inner surface of the ears of rabbits and observing the rapidity of the reaction. A somewhat superior method is to make dilutions of the virus and to note the effect of these dilutions when inoculated on the ears of rabbits. A potent virus should produce vesicles in a dilution of 1 to 500. Efforts have been made to secure a virus in purer form and Noguchi has planted the virus in the testicles of rabbits and of bulls. Virus recovered from this situation is not subject to contamination in the same way as that obtained from surface inoculations. The amount of material obtained, however, is small and the method has not been used extensively enough to justify an opinion as to its value. After preparation of a virus the date should be indicated on the container and the material preserved in the ice chest.

Methods of Inoculation in Man.—As a rule, vaccination is applied on the upper arm over the point of insertion of the deltoid muscle. This situation offers protection against injury and contamination such as is not afforded by vaccination upon the leg or thigh. The area is carefully cleansed with soap and water, followed by alcohol or ether and then by distilled water. The last step is sometimes omitted. Formerly the area was scarified in a criss-cross manner by means of a needle or scalpel, but such extensive scarification has been found to be unnecessary and also exposes a greater surface to the possibility of infection. The more modern method is to place the virus upon the area and to make a scarification through the virus. This may be done by a small linear incision, by the drill method or by the multiple puncture method. Wright has advised intracutaneous inoculation.

Method of Linear Incision.—After placing the virus upon the skin a sterile needle or a small scalpel is employed for making a scarification through the virus and sufficiently deep into the skin to permit absorption but not to produce bleeding. The virus is then gently rubbed into the abrasion and permitted to dry. If a dressing is desired it should be of sterile gauze loosely applied with adhesive strips after the virus has completely dried. Sealing with collodion should not be attempted, since it may permit more ready growth of contaminating bacteria and produce maceration of the skin.

The Drill Method.—A sterile drill such as is employed in the Von Pirquet cutaneous tuberculin test is held between the thumb and middle finger. With a twisting motion and moderately firm pressure a small abrasion the diameter of the drill is made through the virus. This should penetrate the epiderm, but should draw no blood.

The Multiple Puncture Method.—A sterile needle is held nearly parallel with the skin and the point placed through a drop of virus so as

to make an oblique puncture of the epidermis. This is repeated so as to produce about six radially disposed punctures, the whole area extending not more than about 5 mm.

The Intracutaneous Method.—The virus is diluted to ten times its volume with distilled water and injected intracutaneously by means of a sterile tuberculin syringe and a fine needle. Two injections about 2 cm. apart are made.

All the methods indicated have given equally good results, but convenience usually dictates the use of the linear incision or the drill method. It is not uncommon in the use of any of these methods to make two or three inoculations.

Vaccinia.—Following the inoculation of the virus the areas usually remain quiescent for from two to four days when slight reddening and itching may develop. Following this a small papule appears, rapidly succeeded by the vesicle. It is important to note that the vesicle is umbilicated and that its multilocular character is indicated by the minute vesicular arrangement of the margin. The vesicle appears in from five to six days, rapidly becomes pustular and is followed by the formation of the crust. The crust is allowed to drop off and subsequent observations of the scar should show a smooth center, a somewhat scalloped edge and more or less discrete minute marginal scars. During the height of the local reaction the patient may complain of malaise, headache, fever, constipation and other general symptoms. The reaction of vaccinoid has been discussed in the chapter on Hypersusceptibility (page 243).

Immunity as the Result of Vaccination.—The extent of immunity has been indicated by the decrease in prevalence of smallpox since the introduction of vaccination. It may also be measured by the success of subsequent vaccinations. Kitasato has revaccinated a series of 931 cases with successful results as follows:

After 1 year 14 per cent.	After 6 years, 64 per cent.
After 2 years 33 per cent.	After 7 years 73 per cent.
After 3 years 47 per cent.	After 8 years 80 per cent.
After 4 years 57 per cent.	After 9 years 85 per cent.
After 5 years 51 per cent.	After 10 years 89 per cent.

It will thus be seen that more than 50 per cent. of individuals are susceptible to revaccination four years after the original vaccination. Millard states that the Government reports of the German Confederacy show 91 per cent. to 93 per cent. successful revaccinations in ten years or more after the primary vaccination and concludes that "immunity acquired through vaccination begins to disappear at about the second year and by the tenth year it disappears almost completely." Other investigators have obtained similar results. King reported that in ninety-six adults who had suffered from smallpox at various ages and showed numerous scars of the disease, vaccination was successful in 75 per cent. These figures indicate that the older conceptions of the

durability of the immunity produced by vaccination are inaccurate. In order to secure satisfactory immunity, vaccination should be repeated at intervals of a few years. In those communities where small-pox is endemic vaccination should be repeated every year. In the presence of epidemics, an unsuccessful vaccination should not be interpreted as indicating immunity and should be repeated at intervals of a week or ten days until successful. We feel that no dependence can be unqualifiedly placed on the signs of immunity as indicated by Force (page 243).

Unfavorable Results of Vaccination.—If human virus be employed the chance of inoculating syphilis must be considered, although the danger is slight. Reports of tetanus following shortly after vaccination have not been particularly well founded and examination of a large number of samples collected by the Hygienic Laboratory in Washington by McCoy and Bengston failed to demonstrate the presence of the bacilli or their spores in filled capillary tubes, seed vaccine or in bulk glycerolated vaccine. "Ivory points" were found to be contaminated as delivered from the manufacturer of the points, as well as after sterilization and charging. McCoy states that "the sale of vaccine virus on or with points in interstate traffic has been prohibited by an order of the Secretary of the Treasury."

The most important source of trouble is the result of vaccination in unclean skin, the use of unclean dressings or other failures of asepsis, more particularly those resulting from carelessness on the part of the patient. Such infections usually remain localized but confuse the interpretation of results and may in rare instances become general infections.

Vaccination Against Rabies.—The cause of rabies is probably a sporozoan parasite discovered by Negri and named by Calkins "*neurocytes hydrophobiae*." Work with this parasite is difficult because of failure to isolate the organism in suitable form. Therefore, the investigations have been conducted with pathological material containing the organism. It is found in greatest amounts in the nervous system and accordingly the brain or cord is selected for experimental work. This material is spoken of as the virus of rabies. Street virus is nerve tissue obtained from an animal suffering with the natural disease. It is extremely variable in virulence, and for this reason is not employed for vaccination of man. Fixed virus is usually the spinal cord of rabbits obtained after a long series of rabbit passages. By these animal passages the virulence increases and the incubation period decreases until a point is reached when the incubation period following inoculation cannot be further shortened. All mammals are susceptible to rabies in different degrees, but birds or reptiles are not susceptible.

The treatment of rabies in man after it has developed has been entirely unsatisfactory by the methods of immunology. Immunization of animals to the rabies virus produces an immune serum capable of killing the virus. Accordingly it was hoped that such a serum could

be employed for human rabies, but results attendant upon this method of treatment have been unsuccessful. Therefore, at the present time, efforts are directed toward producing an active immunity in those who have been exposed to the disease. It is of interest to note that laboratory inoculations in man rarely, if ever, lead to the development of the disease. It is probable that in order for infection to occur the virus must be implanted with animal sputum or some other form of contamination. Bites from rabid dogs are relatively infrequent, and it is therefore unnecessary to immunize an entire population. Furthermore, man is somewhat resistant to infection with rabies. Statistical evidence in regard to the frequency with which rabies follows the bites of rabid dogs are unreliable because of uncertainty as to whether or not the animal was rabid. Doeberl found that in Prussia, where data had been very carefully collected, there was a mortality of 14.8 per cent. in 122 untreated persons bitten by rabid animals between the years 1902 and 1907. Other estimates conform closely to this. More recently, however, Marx has expressed the opinion that the rate of mortality probably does not exceed 6 per cent. to 10 per cent. of untreated bitten persons. The mortality and morbidity rate are practically identical. Fortunately the period of incubation of rabies is of sufficiently long duration so that active immunization may be effected during the period of incubation.

The period of incubation in man is variable and depends to a considerable extent upon the site of the bite or scratch. According to Bauer, the average period of incubation in 510 cases was seventy-two days. In very rare cases the period of incubation may be less than nineteen days and in more rare instances it may be one year or more. Of seventy-three cases of bites about the head and neck the average incubation was fifty-five days; of 144 cases of bites on the upper extremities the average period was eighty-one and one-half days; and of seventeen cases of bites on the lower extremities the average period of incubation was seventy-four days.

Active Immunization. Preparation of Material.—As has been indicated above it is necessary, because of the failure of passive immunization, to produce an active immunization. In spite of the fact that laboratory accidents practically never lead to the development of rabies it is considered dangerous to inoculate man with the living virus. Ferran and subsequently Proescher have, however, employed a method whereby the active fixed virus is employed. Both these investigators stated that no accidents had followed the use of unmodified fixed virus. Högyes has successfully employed dilutions of fresh fixed virus. The majority of investigators, however, have employed virus which has been attenuated by a variety of methods including heat, partial digestion, the action of bile, the action of glycerol, of anti-rabic serum, of phenol and of mechanical disintegration. Nevertheless, the original method of Pasteur is employed almost uniformly throughout the world. For this purpose the virus is passed through rabbits until it acquires

its minimum period of incubation. The material is introduced into the anesthetized rabbit by subdural inoculation. The injection is made through a small trephine opening just back of the eye and to one side of the median line. The injected material is ground with a small quantity of 1 per cent. phenol solution and 0.2 c.c. of this emulsion is injected. After the rabbit is completely paralyzed it is killed with chloroform and the spinal cord removed aseptically. A small ligature is placed around one end of the cord and the cord hung in a sterile bottle in the bottom of which has been placed sticks of potassium hydrate. The bottle is placed in an incubator maintained at 22° to 23° C. Pieces 1. cm. in length are cut off at daily intervals and placed in glycerol where the degree of virulence on that particular day is retained for several weeks. In large laboratories animals may be killed on successive days and the whole cord employed in preparing the material for human protection. In the United States Hygienic Laboratory pieces 0.5 cm. in length emulsified with 2.5 c.c. of salt solution serve for one injection.

Inoculation in Man.—The determination as to who shall receive anti-rabic treatment is often difficult, but skilled veterinarians are able to diagnose rabies in dogs almost invariably. Knowledge of the condition of the animal inflicting a bite is of the utmost importance. Although cats and rats are not uncommonly victims of rabies, this is not frequently a source of infection in man. When a dog bite is received, the animal should be captured and observed for at least two weeks, during which time the symptoms of rabies become manifest. If the animal is killed the brain should be sent in glycerol to the nearest laboratory, where it may be examined for Negri bodies. If these are not found, material should be injected into rabbits. Negative findings in regard to Negri bodies in the dog's brain are not to be accepted as evidence. In our opinion it is wise to administer treatment to all individuals who have been bitten by animals showing any signs of rabies. The material may be supplied to the physician either in the form of small pieces of cord to be emulsified in salt solution or in the form of an emulsion for dilution with salt solution. The injections are given subcutaneously under the skin of the abdomen. If a considerable time has elapsed since the bite or if the bite has been inflicted upon the head or neck the so-called intensive method of treatment is adopted. Under other circumstances the mild treatment may be given. When material is requested from a commercial laboratory or a state laboratory it is necessary to indicate which form of treatment is desired. As an example of the two methods, the scheme of treatment as shown on page 285, adopted by the New York City Board of Health, will serve.

The Effects of Treatment.—Local reactions are frequent and are likely to be severe about the eleventh and nineteenth days of inoculation. These are urticarial in character and the more severe reactions may be accompanied by mild constitutional symptoms. The glycerol contained in the emulsions not infrequently produces severe pain for

a few moments at the site of inoculation. The treatment, although practically safe, is not entirely free from danger. Remlinger in a study of 107,712 cases that had received treatment found forty cases which developed paralysis of the extremities and two of these terminated in death. The cause of this paralysis is not clear. Certain authorities maintain that the virus contains a toxin and that this may lead to lesions of the nerves. The mass of evidence, however, is against rather than in favor of the conception that toxin plays any important part in the virus of rabies. It is also possible that the repeated injections of foreign protein may have some influence. Such accidents are extremely rare and should not interfere with a decision concerning administration of the treatment.

SCHEME OF TREATMENTS.

Day	Mild treatment	Intensive treatment
1st	14 and 13 day cord	12 and 11 day cord, repeat in afternoon
2nd	12 and 11 day cord	10 and 9 day cord; 8 and 7 day cord in afternoon
3rd	10 and 9 day cord	6 day cord
4th	8 and 7 day cord	5 day cord
5th	6 day cord	4 day cord
6th	5 day cord	3 day cord
7th	4 day cord	2 day cord
8th	3 day cord	4 day cord
9th	2 day cord	4 day cord
10th	4 day cord	1 day cord
11th	3 day cord	4 day cord
12th	2 day cord	3 day cord
13th	4 day cord	2 day cord
14th	5 day cord	4 day cord
15th	2 day cord	1 day cord
16th	4 day cord	4 day cord
17th	3 day cord	3 day cord
18th	2 day cord	2 day cord
19th	4 day cord	4 day cord
20th	3 day cord	3 day cord
21st	2 day cord	2 day cord

Results of Treatment.—The benefits of this form of treatment depend to a certain extent upon the time when the injections are begun and also to a certain extent upon the situation of the bite. Granting that fatalities occur in from 6 to 16 per cent. of untreated bitten individuals, the reports of fatalities in from .46 per cent. to 1.25 per cent. of treated cases show markedly beneficial effects. More recent statistics are highly encouraging. During the year 1916 Viala reported that 654 persons were treated at the Pasteur Institute with but one death.

VACCINATION WITH KILLED ORGANISMS

Vaccination Against Typhoid and Paratyphoid Fevers.—Although various investigators had appreciated the possibility of active immunization against typhoid fever, this subject was first placed on a practical basis by Wright in 1896. In the subsequent year Wright and Semple described in detail a satisfactory method for vaccination.

They employed broth cultures of *bacillus typhosus* two to three weeks old, killed by heating to 63° C. for one hour and preserved with 0.5 per cent. phenol. The vaccine was treated for sterility, standardized and employed in doses of 750 to 1000 million organisms. In the same year Pfeiffer and Kolle reported the demonstration of specific antibodies following the immunization of man against the organism. Since that time vaccines have been prepared in a large variety of ways and preventative vaccination is now upon a highly satisfactory basis. Vaccination has been employed in military and civil life and has resulted in a marked decrease in morbidity and mortality. The results obtained in all civilized countries constitutes one of the greatest achievements resulting from the study of immunology.

Preparation of Vaccines.—The organisms may be grown in broth or upon agar. The broth culture or a salt solution suspension of an agar culture may be killed or attenuated. The application of heat or chemicals for the purpose of killing the organisms reduces in a certain measure their antigenic value. If they are dried before being heated, temperatures of 120° to 150° C. reduce the antigenic property very little. Gay, however, points out that the measure of the antigenic value depends upon the determination of different antibodies such as agglutinins and bacteriolysins, but he notes that this offers "an indication rather of the reaction of the animal body than a sure means of determining the degree of protection that has actually been afforded." Numerous investigators have suggested the use of living bacteria, but the knowledge that typhoid fever may exist as a septicemia without intestinal lesions offers an objection to the introduction of living organisms. It has been found extremely difficult to attenuate without killing the bacteria, but it has been recommended that low degrees of temperature, for example 53° C. (Leishman), the use of ether, alcohol, various sugars and other chemical and physical agents may kill the organisms without markedly reducing the antigenic properties. Certain investigators have also suggested the employment of bacterial extracts, but this method has not been widely employed. Gay and his collaborators (page 301) have claimed success in the therapeusis of typhoid fever by the use of sensitized vaccines and have found that active immunization progresses very satisfactorily, according to measurements of specific antibodies, yet this method, if employed for vaccination, is expensive and probably does not give sufficiently superior results to justify its employment in large numbers of individuals.

It is now recognized that the typhoid bacillus may be divided into a number of strains on the basis of cultural and immunological properties. In certain countries, including the United States, a single strain of the organism has been employed for vaccination, but in others a polyvalent vaccine has been employed, the French using ten strains. The organisms are grown on large agar surfaces, emulsified in salt solution and killed by heat. They are then standardized and a preservative, such as phenol, lysol or formaldehyde, added. Twenty-four hours sub-

sequently cultures are made to determine the sterility of the vaccine. Standardization is usually on the basis of 1000 million organisms per cubic centimeter. If it be desired to give a smaller number of organisms, fractions of a cubic centimeter may be employed. It is the practice in commercial houses to place specified doses in small ampoules so that the physician may administer for each dose the contents of a single ampoule. In military practice the vaccine is placed in small bottles with a rubber cap so that a needle may be thrust through the cap and the required amount of vaccine withdrawn into a syringe.

As the paratyphoid fevers have been studied, it has been considered advisable to vaccinate against these at the same time as against typhoid fever. Therefore, vaccines are now prepared containing the bacillus typhosus, bacillus paratyphosus A and bacillus paratyphosus B. It has been customary to introduce smaller quantities of the paratyphoid bacilli so as not to increase to an unfavorable degree the bulk of foreign protein injected. Accordingly for each 1000 million typhoid bacilli there are usually added 500 million each of paratyphoid A and B. The actual numbers, however, vary in different countries. Castellani recommends the addition also of cholera vibrios. This transforms the *triple vaccine* into a *tetra vaccine*. In northern latitudes this is not of particular importance.

As has been indicated, the organisms are usually suspended in salt solution, but recently neutral oil, such as commercial cottonseed oil, has been employed for suspension. For such suspension the organisms must be very carefully dried before being emulsified in the oil. These lipovaccines have the advantage of being administered in one dose and of producing little or no reaction. They produce immunity following a single injection because of the slow absorption of the oil and its contained antigen.

Method of Administration.—In the case of the lipovaccines a single large dose of organisms may be administered. The use of the salt solution suspensions involves several injections. As a rule, the first dose contains 500 million typhoid bacilli and 250 million each of paratyphosus A and B. The second and third doses contain 1000 million typhoid bacilli and 500 million each of the paratyphoid bacilli. The time between injections has been the subject of considerable study, but, as a rule, a period of seven to ten days intervenes between these injections. Subcutaneous administration is practically universal. Intravenous injections have been recommended, but this method is not widely practiced. Lumière and Chevrotier have administered by mouth gelatine-coated pills of a dried mixed polyvalent typhoid colon vaccine. It is probable that this method is not effectual, since the bacterial protein must undergo at least partial digestion in the intestinal tract. Besredka, however, has recently demonstrated in animals the possibility of successful vaccination through the intestinal tract, but his animals had previously been given bile, and it seems likely that this substance produced sufficient lesion of the intestinal mucosa to permit of direct absorption.

Prophylactic Value of Vaccination.—It can readily be understood that the control of individuals in armies offers excellent facilities for determination of the prevalence and mortality of infectious disease. Consequently, much of the statistical evidence favorable to typhoid vaccination has been collected in armies. The following table, taken from Gay, "Typhoid Fever," illustrates the prevalence of typhoid fever in Great Britain and her colonies before vaccination was introduced:

MORBIDITY AND MORTALITY FIGURES FROM TYPHOID FEVER PER 100,000 OF ENGLISH TROOPS IN THE YEAR 1898 IN DIFFERENT LOCALITIES.

Locality	Morbidity	Mortality
Great Britain	120	24
Gibraltar	420	132
South Africa	3290	577
India	3600	1000
Egypt	8100	2340

Gay states that even greater rates of typhoid morbidity have been encountered. The results of anti-typhoid vaccination are splendidly summarized in another table from Gay's work:

RESULTS OF ANTI-TYPHOID IMMUNIZATION IN THE ENGLISH ARMY. MORBIDITY AND MORTALITY PER 100,000.

Locality	Vaccinated			Unvaccinated			Authority
	No.	Morbid.	Mortal.	No.	Morbid.	Mortal.	
India 1900.....	10501	914	161	83135	1665	444	Wright. Leishmann. Firth.
India 1909.....	5473	380	36	6610	2830	390	
India 1910.....	58481	260	29	10794	1390	220	
Various colonies 1913.....	10378	539	40	8936	3040	500	Report of the anti-typhoid committee, London, 1913

The results obtained in the United States Army under the direction of Colonel Russell and his staff have been most impressive. In December of 1919 Colonel Russell summarized the results in a paper in the *Journal of the American Medical Association*. He gives an analysis of a water-borne epidemic in Hawaii as follows:

TYPHOID EPIDEMIC IN HAWAII, H. T., IN THE FALL OF 1917.

	Population on Castner water system	No. of cases of typhoid	Cases per thousand	Deaths		Mortality rate per thousand
				Number	Per cent.	
Vaccinated	4087	55	13.45	4	7.4	0.97
Unvaccinated	812	45	55.41	7	15.5	8.62

It is of importance to note in reading this table the large number of vaccinated as contrasted with the unvaccinated. It is apparent that the vaccinated show not only a reduced morbidity percentage, but also a diminished mortality rate. Colonel Russell gives the following table of figures from the United States Army for nineteen years:

OF TYPHOID FEVER IN THE ARMY AND IN THE CORRESPONDING AGE GROUP
IN CIVIL LIFE FOR THE PAST EIGHTEEN YEARS.

Year	No. of cases	Ratio per thousand	Deaths	Ratio per thousand	Total	Males†
.....	531	5.75	60	0.43	0.46	
.....	594	9.43	78	0.64	0.42	0.54
.....	565	8.58	69	0.86	0.40	
.....	348	5.82	30	0.28	0.35	
.....	247	5.62	12	0.27	0.33	
.....	193	3.57	17	0.30	0.32	
.....	347	5.66	15	0.28	0.32	
.....	208	3.53	16	0.19	0.28	
.....	215	2.94	21	0.23	0.28	
.....	173	3.03	16	0.28	0.23	
.....	142	2.32	10	0.16	0.27	0.34
.....	44	0.85	6	0.09	0.23	
.....	18	0.31	3	0.04	0.18	
.....	4	0.04	0	0.00	0.18	
.....	7	0.07	3	0.03	0.15	
.....	8	0.08	0	0.00	0.18	0.17
.....	25	0.23	3	0.03	0.12	0.15
.....	297	0.44	23	0.03	0.11	0.14
.....	768	0.30	133	0.05	0.09	0.11

Indicates voluntary vaccination against typhoid.

Indicates compulsory vaccination against typhoid.

† Civil deaths from typhoid fever; age group, 20 to 29 years. Rate per thousand of population.

marked change after the introduction of compulsory vaccination in the Army in 1911 is most striking. It is pointed out that the increase in 1917 is in large part contributed to by delay in the vaccination and sanitary control of National Guardsmen. As an impressive contrast the following table illustrates the vast improvement in health conditions as compared with previous wars:

RELATION OF MORTALITY IN THE WORLD WAR TO THAT OF PREVIOUS WARS.

	Number of deaths that occurred in the World War, Sept. 1, 1917- May 2, 1919. Average strength approximately 2,121,396	Number of deaths that would have occurred if the Civil War death rate had obtained	Number of deaths that would have occurred if the Spanish-American War death rate had obtained
Typhoid fever	213	51,133	68,164
Malaria	13	13,951*	11,317
Dysentery	42	63,898†	6,382†

Includes malaria, remittent and congestive fevers.

Includes dysentery and diarrhea.

During the period of the American participation in the World War there were 1065 cases of typhoid fever in approximately 4,000,000 troops, a ratio of one case to every 3756 men. In the Spanish-American War there was one case to every seven men. Colonel Russell's final comment is of the greatest interest. "It is evident from these tables, therefore, that anti-typhoid vaccination, carried out as it was by a personnel which had not been carefully trained in its administration, gave a high degree of protection to our forces under the conditions of hurried mobilization and of warfare, and reduced the rate, not only below

the rates for previous wars, but also below the rate found in civil life in some of the older states where the entire population is protected by all the sanitary measures of modern life."

At the beginning of the World War, of the troops in Belgium only those of the British Army were adequately protected. At the beginning of trench warfare in 1914 an epidemic broke out, and in January and February of 1915, 4000 cases occurred in Dunkirk. Up to May of 1915 only 827 cases were contributed from the British Army, the bulk of the cases came from among the unvaccinated Belgian soldiers and civilians. Vaccination was instituted in February, and the epidemic was at an end by the middle of the summer. In the early days of the war vaccination had not been compulsory in the French Army, and as the result a large number of troops were victims of typhoid fever. The institution of vaccination completely altered the picture. Courmont gives the following statistics for the French Army in 1916:

	Deaths
Non-vaccinated cases	17.4 per cent.
Of the vaccinated cases:	
Those who had one injection	6.0 per cent.
Those who had two injections	4.0 per cent.
Those who had three injections	2.5 per cent.
Those who had four injections	1.9 per cent.

Duration of Protection.—When typhoid vaccination was first introduced it was generally assumed that protection lasts for about two years. Certain British troops in Mudros were found to have developed typhoid fever within six months after inoculation. Similarly, certain troops of the American Army developed typhoid fever a few months after they had been vaccinated, but it was found upon investigation that in this instance the vaccination had not been completed. On the basis of experience, yearly vaccinations were practiced in the British Army, although it was not considered necessary to give the three doses at the time of revaccination; a single maximum dose on revaccination apparently served to maintain immunity. Yearly revaccination, however, provides adequate protection. Knowing that infection has occurred within a few months after proper vaccination it is no longer advisable to state that protection lasts for more than a year. The determination as to when revaccination must be practiced depends in certain measure upon the degree of exposure to the disease. In those districts where typhoid or paratyphoid fevers are endemic, we recommend that vaccination be reinforced by a single yearly inoculation of the maximum dose. If a period of two years has elapsed since previous vaccination, it is advisable to revaccinate with three injections.

Complications.—The reaction to any dose of typhoid vaccine is extremely variable. Usually the second and third doses produce somewhat more severe reactions than the first dose. There are, however, certain individuals who are apparently hypersusceptible to typhoid protein, and these may react with great severity. As a rule, reactions are merely local and are exhibited by swelling, redness, tenderness and pain about the site of inoculation. General reactions are much less fre-

quent and are exhibited by malaise, headache, fever, constipation and occasionally chills. Maurange investigated the general reaction following 39,215 inoculations and reports the following results:

Types of reaction	Typhoid per cent.	Para bacilli per cent.
None	92.23	98.59
Feeble	6.18	1.41
Moderate	1.40	0.00
Pronounced	0.19	0.00

Rarely the inoculations may be followed by arthritis, nephritis and severe intestinal disturbances. Chantemesse has called attention to the recrudescence of tuberculosis during immunization, and it has further been suggested that other chronic diseases, including syphilis, may be excited to renewed activity. We have observed cardiac arrhythmia in individuals who have previously suffered from myocardial disease.

Contraindications.—The contraindications include kidney disease, diabetes, myocardial and endocardial disease, aortitis, cachexia, gastrointestinal disturbances and alcoholism. The presence of acute infections is also regarded as contraindicating vaccination. According to Maurange, age is no contraindication, although the relative unsusceptibility of old people to typhoid fever may make it seem unnecessary to vaccinate. Menstruation is not a contraindication.

Vaccination Against Cholera.—This was first employed by Ferran in 1884. Haffkine published results in 1888, and since then numerous investigations have developed technical methods and have emphasized the value of protective vaccination. Ferran injected broth cultures of living vibrios subcutaneously, employing 0.25 c.c. as the first dose and 0.5 c.c. as the second and third doses. Haffkine employed two kinds of vaccine, a weaker vaccine prepared from living organisms grown on agar at 39° C. and a more virulent vaccine prepared from vibrios which had been passed through a series of guinea-pigs. Subsequently Kolle prepared a vaccine made from heat-killed agar cultures of virulent organisms. The emulsion is made by suspending 2 mg. organisms in saline and heating to 60° C. for one hour. Cantacuzène prepared a vaccine by heating emulsions of the vibrios for one and one-half hours at 55° to 56° C. The concentration of this vaccine was 500 to 1000 million organisms per cubic centimeter. Two inoculations were given, the first of 2 c.c. and the second of 4 c.c. with a six-day interval. Strong prepared a vaccine by incubating the emulsion in sterile water, thereby breaking up the cells. The emulsion was then passed through a Reichel filter and the sterile filtrate employed. At the present day heat-killed vaccines are most commonly employed.

Results.—The earlier work of Ferran and of Haffkine was extremely encouraging, but the subsequent statistics lend even greater support to the value of this procedure. Arnaud made a study of 108,000 men during the second Balkan War. These men were all in infected areas. Among the unvaccinated men the morbidity was 5.75 per cent. Among those who had received insufficient vaccination it was 3.12 per cent., and among those who had received the full treatment it

was 0.41 per cent. Kobe made an extensive study of the population of Tokio and its suburbs. In the city of Tokio, 10.54 per cent. of the entire population were vaccinated. The absolute number that were vaccinated, namely, 238,936 in Tokio and 61,988 in the suburbs, as well as the large number of controls, provides a sufficient number from which to draw satisfactory conclusions. In Tokio cholera occurred in 1.85 per 10,000 of the unvaccinated and 0.13 per 10,000 among the vaccinated. In the suburbs cholera occurred in 3.09 per 10,000 of the unvaccinated, and there were no cases reported among the vaccinated. Cantacuzène has studied results obtained in the campaigns in the Orient during the Balkan Wars and the World War. These were conducted particularly during the epidemics, and by a study of the normal curve of epidemics he finds that vaccination leads to a sharp drop in the epidemic curve and incidence of the disease.

Vaccination Against Pneumonia.—Although vaccination against pneumonia was practiced by Wright before the various types of pneumococci had been identified, it was not until the types were carefully studied that exact results could be obtained. Lister, after he had identified the types of organisms present in South Africa, carried out prophylactic immunization in 11,000 workers in the Rand mines. He employed a composite vaccine prepared from the pneumococcus types prevalent in that region. He found that subcutaneous inoculations were sufficient to establish an immunity, and demonstrated that the protection was effective against the particular type of pneumococcus used in the vaccines. He emphasized the importance of using a large bulk of organisms and considers that the minimum effective dose is at least 6000 million pneumococci of each group against which protection is sought. The work of Cecil and Austin and of Cecil and Vaughan has been of the utmost importance. Cecil and Austin employed a saline suspension of killed pneumococci of Types I, II and III. Three or four doses were given at intervals of five to seven days. The first dose contained 1000 million of each of the three types; the second contained 2000 million of each type, and the third and fourth contained 3000 million each of Types I and II and 1500 million Type III. At Camp Upton 12,000 troops were vaccinated, and among these only seventeen cases of pneumonia of all types developed, including those due to Type IV as well as to the streptococcus. Among the 20,000 unvaccinated men, 172 cases were reported. At Camp Wheeler a lipovaccine was employed containing 10,000 million each of Types I, II and III per cubic centimeter, given in one dose. Eighty per cent. of the total command, or 13,460 men, were vaccinated and 363 cases of pneumonia of all varieties developed. The study was difficult because of the prevailing influenza epidemic. An analysis of the records shows that "there were thirty-two cases of Types I, II and III pneumonia among the vaccinated four-fifths of camp, and forty-two cases of pneumonia of these types among the unvaccinated one-fifth of camp. If, however, all cases of pneumonia that developed within one week after vaccination are excluded from the vaccinated group, there remain only eight cases of

pneumonia produced by fixed types, and these were all secondary to severe attacks of influenza. This exclusion is justified by the fact that protective bodies do not begin to appear in the serum until the eighth day after injection of pneumococcus lipovaccine." "The pneumonia incidence rate per 1000 men during the period of the experiment was twice as high for unvaccinated recruits as for vaccinated recruits, and nearly seven times as high for unvaccinated seasoned men as for vaccinated seasoned men." The death rate for vaccinated men, in whom the pneumonia developed more than one week after vaccination was 12.2 per cent., whereas among the unvaccinated troops it was 22.3 per cent. The death rate for primary pneumonias was only one-third as great among vaccinated men as among unvaccinated, but the rate in pneumonia secondary to influenza was about the same for both groups. Among the 20 per cent. of the command which were unvaccinated 327 cases were reported. These statistics were sufficiently encouraging to introduce vaccination into the army on a fairly large scale. Nevertheless, the results are not sufficiently conclusive to state positively that a high degree of protection is obtained. Recently Cecil and Blake have been able to produce in monkeys a characteristic pneumococcus pneumonia by intratracheal inoculation. These investigators have studied the problem of vaccination with saline vaccines and lipovaccines of killed pneumococci and in addition have investigated the value of vaccination with living organisms. They found that vaccination with killed pneumococci was not effective in preventing the development of the disease under the conditions of infection but that the vaccinated animals showed a somewhat less severe form of the disease. The living vaccine was considerably more satisfactory, but they state that "the method is too dangerous for any sort of practical application." Vaccination with living virulent pneumococcus, Type I, produces a protective immunity against pneumonia of homologous types. The immunity against other types of pneumococcus following vaccination with Type I offers a certain degree of protection against other types, but this varies considerably with the individual monkey. Vaccination with "living avirulent pneumococcus Type I, if administered in sufficiently large doses, renders the monkey immune to a subsequent pneumonia of homologous type." Cecil and Blake point out that "vaccination with attenuated living pneumococci could probably be practiced with impunity, but the problem of transporting and keeping alive large quantities of pneumococci in the field would be difficult to solve."

Vaccination Against Plague.—Prophylactic vaccination against plague was first reported by Haffkine in 1897. Subsequently Pfeiffer and also Gaffky reported experiments which support the value of this type of vaccination. Haffkine's vaccine was prepared from a killed broth culture of the bacillus pestis five or six weeks old. Adult males were given 3 to 3.5 c.c. and adult females 2 to 2.5 c.c. Kolle's vaccine is prepared from slant agar growths suspended in the proportion of 2 mg. of bacilli to the cubic centimeter of salt solution. Kolle and Strong also employed living organisms whose virulence had been greatly re-

duced. Lustig and Galeotti used nucleoprotein extracted from the organisms and Kitano and others have employed organisms grown in Bengal isinglass medium. Kitano and Sukegawa employed sensitized vaccines and are of the opinion that these give better results than the usual heated vaccine. They gave in the first dose 2 mg. of the sensitized organism and in the second dose 4 mg. of the sensitized organism. If haste is essential, 6 mg. may be given at one dose.

Experimentally, it has been established that vaccinated animals display an increased resistance against the disease. The Indian Plague Commission reported that vaccination in man diminishes the incidence of the disease, but that it does not furnish absolute protection. Apparently the duration of immunity lasts from a few weeks to a few months, but immune bodies are not demonstrable until ten days have elapsed. In spite of the fact that numerous investigators have reported favorably on vaccination against plague, Flu has stated that an analysis of the statistics fails to furnish evidence that sufficient attention has been given in the earlier studies to the prevalence of infected rats or to other hygienic conditions which prevailed.

Vaccination Against Typhus Fever.—Vaccination against this disease has been attempted with the serum of convalescent patients, but the results have not been highly satisfactory. Plotz, Olitsky and Baehr employed a vaccine composed of fifteen strains of bacillus typhi exanthematici. Of a series of 5251 vaccinated individuals where typhus was epidemic only three contracted the disease, and in another series of 8420 cases only six contracted the disease. Although the work of Plotz with this organism has been carefully done there is still doubt as to its exact etiological relationship. In statistics concerning this disease, the presence of infected lice should be taken carefully into consideration. It cannot be stated that vaccination in typhus has any great value until further investigations have been conducted.

Vaccination Against Pertussis (Whooping-Cough).—The discovery of the bacillus of whooping-cough by Gengou almost immediately led to investigation as to vaccination. Luttinger has summarized the results obtained in a large whooping-cough clinic and by over 180 private physicians and health officers. The results were sufficiently encouraging to justify the recommendation of this procedure. Conditions of exposure and the nature of surroundings, as well as the variability of the disease, even in a single epidemic, makes the interpretation of statistics extremely difficult.

Vaccination Against Dysentery.—Prophylactic vaccination against dysentery has encountered great difficulties because of the extreme toxicity of the cultures. Shiga attempted to overcome this by employing mixed active and passive immunization. He used a bacterial vaccine to which was added immune serum. Experiments on 10,000 individuals showed a definite decrease in the rate of mortality. Others have employed toxin-antitoxin mixtures with apparent success, but Hoffmann found that this type of vaccination failed to have any effect on the control of a dysentery epidemic which he studied. Whitmore and Fennel and

also Fennel and Petersen have prepared lipovaccines. It was found possible to administer in a single dose 3000 million Shiga organisms, 3200 million Y type organisms and 2200 million Flexner organisms without marked local or general reaction. In experiments with animals immune sera can be prepared with much less difficulty when the organisms are administered suspended in oil. The method has not as yet been given sufficiently extensive trials in man to justify definite statements as to its efficacy, but from the experimental results obtained it appears to have more promise than any of the other methods proposed.

Vaccination Against Influenza.—Vaccination against influenza was practiced very extensively in the recent great epidemic. The controversy over the etiological relationship of the bacillus of Pfeiffer has, in our opinion, not been settled. The results of vaccination with this organism might serve to settle in part the question as to the cause of the disease, since a high degree of immunity to the disease following vaccination, if interpreted in the sense of specificity, would indicate that the organism employed is the exciting cause. The vaccines which have been employed have been suspensions in salt solution, killed by heat. In certain districts stock cultures have been employed, in others a culture of a strain or strains isolated during the epidemic has been used, and in still others a mixed vaccine has been used composed of the bacillus of Pfeiffer, the streptococcus, the pneumococcus, the staphylococcus and other organisms. Reports of striking success following vaccination have been numerous, including in particular the work of Duval and his collaborators. In consideration of reports of this sort the curve of the epidemic has sometimes been overlooked. Reports of certain other investigators have not been encouraging. McCoy states that "the general impression gained from uncontrolled use of vaccines is that they are of value in the prevention of influenza; but, in every case in which vaccines have been tried under perfectly-controlled conditions, they have failed to influence in a definite manner either the morbidity or the mortality." At best the method must be regarded as still in the experimental stage.

Vaccination Against Other Diseases.—Vaccines have been prepared against scarlatina, cerebrospinal meningitis, tuberculosis and contaminated wounds. Examination of the statistics presented fails to produce convincing evidence that vaccination against these conditions is especially satisfactory. As time goes on, methods may be improved and larger statistical evidence collected.

APPENDIX C

VACCINE THERAPY

INTRODUCTION.
DISEASES OF THE GENITO-URINARY TRACT.
GONORRHEA.
CYSTITIS.
PYELITIS AND SUPPURATIVE NEPHRITIS.
DISEASES OF THE SKIN.
FURUNCULOSIS.
CARBUNCLES.
ECZEMA.
RINGWORM.
OTHER SKIN DISEASES.
DISEASES OF THE RESPIRATORY TRACT.
RHINITIS.
OZENA.
ASTHMA.
PERTUSSIS.
PNEUMONIA.
OTHER DISEASES.
DISEASES OF THE EYE.
DISEASES OF THE ALIMENTARY CANAL.
TYPHOID FEVER.
PARATYPHOID FEVER.
DYSENTERY.
TUBERCULOSIS.

VACCINE THERAPY

Introduction.—A clear differentiation must be made between prophylactic vaccination and therapeutic vaccination. The value of various modes of prophylactic vaccination has been discussed and their importance in protection against various diseases has been outlined. For purposes of discussion of therapeutic vaccination it is well to consider the infectious diseases as either acute or chronic and either local or general. Acute infectious processes are for the most part self-limited and require little in the way of specific treatment, and spontaneous cure is so regular as to render difficult the interpretation of results following therapeutic vaccination. Chronic infectious diseases tend to be progressive and finally result either directly or indirectly in the death of the patient. Statistical reports may show instances of amelioration of the disease, but the personal bias of the investigator may sometimes confuse the conclusion. Generalized infections may be treated by simple bacterial vaccination, but the results with sensitized vaccines have been better than those with unsensitized vaccines. With few exceptions the results of therapeutic vaccination have been best in cases of localized infection. The vaccines employed may be in the form of stock vaccines, but the opinion is practically universal that wherever possible the employment of autogenous vaccines gives the best results.

The persistence of chronic infections is, in part, due to the fact that the chronic inflammatory fibrous tissue hinders the general absorption of antigenic materials produced by the exciting organism. Conse-

quently, immune bodies are not produced in sufficient amounts to combat the infection. Vaccination may serve to stimulate a general immune reaction which aids in the resistance to the local lesion. In generalized infections the simple bacterial vaccines may add to the load carried by the body and perhaps reduce rather than enhance immunity. If, however, immune serum is added to the vaccine or introduced separately, the serum may operate either upon the body or upon the bacteria so as to favor resistance.

DISEASES OF THE GENITO-URINARY TRACT

Vaccine Treatment of Gonorrhea.—If stock vaccines are to be employed, it is desirable to use those composed of a variety of strains of the organisms. Many of the vaccines employed are heated salt solution suspensions of the organisms. Démonchy advises the use of large doses of unheated salt solution suspensions of stock cultures. Thomson has prepared a so-called detoxicated vaccine. In the earlier method Thomson dissolved the organism in N/10 NaOH and precipitated with N.HCl. The toxins remain in the supernatant fluid. Later he found that the toxins could be removed by washing with 0.5 per cent. sodium acid phosphate and 0.5 per cent. phenol. Haworth employed sensitized vaccine, and recently Sézary has recommended lipovaccine. Most investigators recommend the employment of large doses of the organisms, ranging from a minimum of 5000 million to a maximum of 25,000 million.

The vaccines have been employed in acute gonorrheal urethritis but with relatively little success. They have also been employed in vulvo-vaginitis in children, in some instances with apparent success. Undoubtedly, the field for therapeutics of this sort is best realized in gonorrheal arthritis. In this condition persistent vaccination has been followed in many cases by excellent results. Somewhat similar are the chronic infections of urethral glands, prostate, seminal vesicles and the internal female genitalia. Results from treatment of these conditions warrant a trial of vaccine treatment in conjunction with other modes of treatment or in those instances where other forms of treatment have failed or are contraindicated.

Cystitis.—The organisms which may cause cystitis are variable, but in those cases where the disease is chronic and resistant to local treatment the causative organism usually belongs in the colon typhoid group, the bacillus coli communis being the most frequent offender. In treatment of this disease it is of fundamental importance to discover the cause. In cases due to the colon bacillus the vaccine may be given in the form of killed salt solution suspensions of organisms isolated from the case. Stock vaccines may be employed when necessary. The dose is usually from 50 million to 100 million. Results have been extremely variable, but the method is sufficiently well established to justify trial in resistant cases. Of fundamental importance is the removal of urethral stricture, prolapse of the bladder or other local conditions which retard cure.

Pyelitis and Suppurative Nephritis.—In pyelitis the causative organism should be discovered before vaccine treatment is considered. If due to the *bacillus coli communis*, autogenous vaccines in doses of from 50 million to 100 million organisms given at weekly intervals often yield good results. Suppurative nephritis occasionally is improved by vaccination with the causative organism, but the danger of widespread infection as a result of the disease is so great that in our opinion surgical measures are of more immediate importance unless the general condition of the patient contraindicates operation.

DISEASES OF THE SKIN

Many of the diseases of the skin and of the subcutaneous tissues depend upon the local action of bacteria; a considerable number of these is susceptible to vaccine treatment. A greater number of skin diseases is the result of more deep seated disorders and under these circumstances it is essential that the cause be corrected; in these instances vaccine treatment is of little avail unless the primary disease is one susceptible to that mode of treatment.

Furunculosis.—Furuncles are usually caused by some variety of the staphylococcus, most frequently the *staphylococcus pyogenes aureus*. Occasionally, furuncles may be the result of streptococcus infections or of mixed infections. The single furuncle usually heals after the pus is discharged, either naturally or surgically, and may clear up without any interference whatever. Patients are seen, however, in whom furuncles appear repeatedly. In some of these cases the underlying cause is diabetes mellitus and in others it is apparently due to a prolonged decrease in the number of circulating leucocytes. Vaccination in cases in which the boils are persistent and frequent is usually effective. Stock vaccines are frequently employed, but in this condition, as in others, autogenous vaccines are to be preferred. Stock vaccines have frequently failed because of failure to identify the exact organism causing the condition. For example, *staphylococcus aureus* stock vaccines are employed on the assumption that the boils are due to this organism, whereas if cultures were made from the boil another organism might be isolated. It is generally recommended that the vaccine be composed of 2000 million organisms per cubic centimeter. It is important that the first dose be relatively small and the increase in doses gradual. At the first dose 0.1 c.c. is given and at the second dose 0.2 c.c. is given and the doses increase by gradations of 0.1 c.c. until the maximum dose of 1.0 c.c. is reached. It is often recommended that the doses be given eight days apart, but this period may be reduced with advantage to three or four days. In case of diabetes the vaccination should proceed more slowly and with somewhat smaller doses than in other cases. With the dosage recommended, local reactions are slight and general reactions very rarely appear. Vaccination in furunculosis usually gives excellent results and is to be highly recommended.

Carbuncles.—These are also benefited in certain instances by vac-

cine treatment, but it must be expected that many cases will fail to improve. On the whole, surgical treatment is more satisfactory.

Eczema.—The recent studies of this disease have shown that many cases are the result of hypersusceptibility to proteins, usually those contained in food. Granted that such hypersusceptibility is demonstrable, treatment is in the form of immunization to the particular protein concerned. Such immunization is similar to that employed in hay fever and has been commented on in the chapter on hypersusceptibility (page 231). Kolmer states that the prolonged administration of an autogenous bacterial vaccine composed of staphylococci procured from the scales or serous exudate has occasionally aided in the treatment of obstinate cases of eczema.

Ringworm.—Strickler has recently employed a vaccine made of several strains of the fungus and is of the opinion that the method has some value in obstinate cases.

Other Skin Diseases.—Vaccination has been employed with a variable degree of success in the different forms of acne, sycosis, scrofuloderma, impetigo and certain forms of erythema.

DISEASES OF THE RESPIRATORY TRACT

Rhinitis.—Vaccination against acute rhinitis has been largely prophylactic in nature, and the results of these vaccinations have been in a general way favorable. The exact cause of this disease has not been finally proven, but the work of Foster indicates rather strongly that the agent is a filterable virus. The prophylactic vaccines, however, have been mixed stock vaccines of a variety of bacteria, and it seems probable to us that any success obtained upon this basis is probably non-specific. Coates is of the opinion that if acute rhinitis is treated early with vaccines there is likely to be improvement. The course of acute rhinitis is so variable that statistical results are open to some question. In chronic rhinitis it is maintained that autogenous vaccines are of value. It must be understood, however, that contributory causes, such as adenoids, enlarged tonsils, polyps and nasal deformities must be removed. So much benefit accrues from the correcting of the contributory causes that the beneficial effects of vaccination probably depend in certain part upon the personal equation of the observer.

Ozena.—The cause of this disease is at present a matter of considerable dispute, and the value of vaccination is undecided. The vaccines that have been employed are usually made from the bacillus *ozenæ fetidæ* of Perez. Horn claims that this organism is similar to the bacillus *bronchisepticus* and has made polyvalent stock vaccines which he claims are highly successful. Friel reports excellent results from the intravenous administration of sensitized living vaccine of Friedländer bacillus. Ersner has had disappointing results. While improvement may occur in a certain percentage of cases, McKenzie found a marked tendency to relapse following the cessation of treatment.

Asthma.—As with eczema, the recent investigations of hypersusceptibility have placed the study of asthma upon an entirely new

basis. According to the work of Walker and his collaborators, certain of the cases are due to specific bacterial invasion, and probably contributed to by a certain degree of hypersusceptibility to the organism. Bacterial vaccination in these cases has been accompanied by good results. A complete investigation of the nature of the case is essential before any form of vaccine treatment should be attempted. Earlier investigators have employed mixed vaccines made of organisms obtained from the sputum.

Pertussis.—Prophylactic vaccination against this disease has been discussed (page 294). Therapeutic vaccination has been employed by a number of workers with, in many instances, apparently favorable results. Luttinger found that in a series of 952 cases treated by vaccination the paroxysmal stage averaged about thirty-seven days, whereas 149 cases not treated with vaccine had a duration of over fifty days. Blum and Smith found that non-specific vaccination was practically as effective as vaccination with the bacillus of Gengou. Barenberg also finds that pertussis vaccine, even when given in large doses, has neither curative nor ameliorating effect. Kraus and others have reported good results by the use of a vaccine prepared from the sputum. The sputum is washed, mixed with ether, shaken for three or four days, the ether evaporated, the mixture tested for sterility and given in doses of 1.0 c.c. every three or four days. If stock vaccines of the organisms are employed, it is of the utmost importance that they be fresh. Doses of 25 million organisms may safely be given.

Pneumonia.—Prophylactic vaccination (page 292) is distinctly more promising than therapeutic vaccination. Treatment with immune serum (page 256) is also more promising than vaccination. Coleman is of the opinion that vaccines in pneumonia are never harmful and may be beneficial. Teale and Embleton believe that they have obtained good results in certain cases. Shera expresses the belief that the local infection is too massive to permit of vaccine having any appreciable effect in the stage of consolidation. The frequent occurrence of pneumococcus septicemia as a part of the disease makes it unlikely that vaccination will be helpful. In delayed resolution vaccines are of value in some cases. Shera also states that empyema when it has reached the chronic stage may be benefited by specific vaccination.

Other Diseases.—Certain diseases of the accessory regions of the respiratory tract, including chronic median otitis and mastoiditis, have been treated by vaccination with the organism concerned. Results have been variable, but inasmuch as these represent somewhat isolated local infections it is reasonable to attempt vaccination in addition to the usual modes of treatment.

DISEASES OF THE EYE

When conjunctivitis becomes chronic, specific vaccination sometimes leads to improvement. The infecting agents include staphylococcus, streptococcus, bacillus pyocyaneus, Friedländer's bacillus and others. Autogenous vaccines may be employed in addition to other modes of

treatment. Chronic conjunctivitis, due to the Morax-Axenfeld diplobacillus, is said to respond very well to vaccine treatment. In acute pneumococcus and gonococcus conjunctivitis, especially with ulcer, Allen advises early and vigorous vaccine therapy and reports good results. It is also stated that ophthalmia neonatorum sometimes improves rapidly under vaccine treatment. In none of these conditions, however, is it wise to neglect other forms of treatment.

DISEASES OF THE ALIMENTARY CANAL

Typhoid Fever.—Prophylactic vaccination has unquestioned value in the prevention of typhoid fever (page 285). Specific therapeutic vaccination has been the subject of experiment since the work of Fraenkel in 1893. The vaccines employed have been usually killed organisms either untreated or sensitized, administered either subcutaneously or intravenously. Certain authors have also reported the use of living organisms, but this method has not been adopted. Gay, in his book, "Typhoid Fever," reports the following summary of results obtained by various methods:

SUMMARY OF RESULTS OBTAINED BY RECENT OBSERVERS (1913-1917) IN THE TREATMENT OF TYPHOID FEVER BY VACCINES ADMINISTERED IN VARIOUS WAYS.

	Observers	Total cases	Estimates based on	Benefited	Mortality
Untreated vaccine subcutaneously	30	1001	512	46%	14.5%
Sensitized vaccine subcutaneously	14	593	239	69%	8.0%
Untreated vaccine intravenously	22	501	233	62%	13.0%
Sensitized vaccine intravenously	12	487	316	85%	11.0%

It is usually stated that typhoid fever has a mortality of about 10 per cent., although in the American Civil War it exceeded 35 per cent. and in the Franco-Prussian, Spanish-American and Boer Wars it ranged between 8 and 14 per cent. The severity of epidemics varies considerably, but at the best there is little in the way of encouragement to be found in the table given above. The basis upon which improvement is estimated varies considerably with the different investigators and the figures are "distinctly affected by subjective influences." Gay has employed a sensitized vaccine administered intravenously and his results in ninety-eight cases are summarized as follows:

SUMMARY OF RESULTS IN NINETY-EIGHT CASES OF TYPHOID TREATED BY INTRAVENOUS INJECTION OF SENSITIZED VACCINE SEDIMENT.

	No. of cases	Age	Widal titer on beginning treatment	Blood culture positive	Treatment begun day	No. of treatments	Permanent normal	Days of treatment
Aborted	33	26.2	296.0	36.6%	13.4	1.88	20.4	7.0
Benefited	32	24.2	156.5	70.9%	14.8	3.20	30.6	15.8
Unaffected ...	33	28.8	114.8	84.8%	13.7	4.85	46.8	33.1

The most significant figures in this table refer to those cases which were aborted. Careful study of various epidemics fails to show any instance where such a large percentage of the cases have aborted, and it therefore seems probable that the vaccination had some distinct value.

This rapid improvement appeared to be somewhat more striking in the moderate and mild cases than in those which were considered severe.

Other investigators have noted that non-specific therapy has been quite as effective as the use of specific typhoid vaccine. Kraus found that colon bacilli were equally effective and others have confirmed this observation. Lüdke has employed deuterio-albumose, Weichardt albumin solutions, Nolf pepton and still others have employed such substances as dextrose, colloidal gold and even normal salt solution. Gay admits that the non-specific form of therapy has been as effective as the use of sensitized typhoid vaccines, but urges the employment of typhoid vaccine because it may be kept indefinitely in dried form under conditions of strict asepsis and can readily be injected in exact amounts. He further states that "typhoid vaccine has the advantage over other protein preparations of building up the active immunity of the patient, and a sensitized vaccine will, in our experience, produce a higher grade of leucocytosis."

Paratyphoid Fever.—Rathery and others have used therapeutic vaccination in paratyphoid B fever. It was concluded that the treatment is useful, always improves general condition, often shortens the fever and has never led to harmful results. Others have found that typhoid vaccine is as effective in paratyphoid as in true typhoid fever and the non-specific therapy indicated above has also been effective.

Dysentery.—The vaccine treatment of dysentery is confined to the bacillary form and of these varieties the cases due to the Flexner bacillus and other related forms appear to do much better than those caused by the Shiga bacillus. Nolf, from his observations in the Belgian Army, concludes that vaccine therapy, when administered by the intravenous route is the most effective therapeutic procedure in the more chronic forms of bacillary dysentery. His cases did not include those caused by the Shiga bacillus. Similar results had been reported by Baroni in the Roumanian Army. He employed either six injections of killed organisms or four injections of living vaccine. Kountze found that in typical cases of dysentery, vaccination produced immediate general improvement and reduction in the number of stools. The study of the therapy of this disease has been somewhat hampered by the failure of investigators to identify the strains of organisms concerned. Although the results with vaccination have been encouraging, it is by no means positively proven that this mode of treatment is superior to serum treatment.

TUBERCULOSIS

The various forms of tuberculin are vaccines and treatment by their use is an example of vaccine therapy. The methods of preparation of the various tuberculins have been discussed (page 238). Koch's first work with tuberculins was stimulated by the hope that treatment with them might be effective. The use of the material in larger amounts than now seem necessary led to severe reactions on the part of the patients which in some instances were disastrous. For many years tuberculin therapy was considered extremely dangerous and was practiced by very few clinicians. Recently, however, a more thorough

knowledge of the proper precautions in treatment has been built up and satisfactory results are now reported. Applied to pulmonary tuberculosis it has been followed by improvement in many cases, particularly in those under sanitarium treatment. It also is claimed to be an important aid in the treatment of tuberculosis of the bones and joints and of the eye. Improvement has been reported also in cases of tuberculous enteritis and mesenteric lymphadenitis. Kleinberg, however, maintains that only a small proportion of bone and joint cases improve, that the majority show no improvement and that in some cases relapses occurred and new abscesses appeared.

Apparently the most suitable patients for tuberculin therapy are those with incipient tuberculosis or old cases of fibroid phthisis with fair or good nutrition. Advanced or moderately advanced cases may be so treated if the general condition is good. Hamman and Wolman do not consider marked general weakness, fever, cardiac disease, nephritis, epilepsy, syphilis of themselves contraindications but rather unfortunate complications which may prevent specific treatment.

The injections are given subcutaneously at the lower angle of the scapula. In order to observe whether or not reaction occurs the injections are given in the afternoon after the patient's temperature has been taken. This avoids mistaking an accidental afternoon rise of temperature for a rise due to the tuberculin. Hamman and Wolman recommend the following range of doses:

Tuberculin	Initial dose	Maximal dose
Old tuberculin	0.000,000,1 to 0.000,001 c.c.	1. c.c.
New tuberculin	0.000,001 to 0.000,1 c.c.	2. c.c.
Bacillus emulsion	0.000,001 to 0.000,1 c.c.	2. c.c.

Three classes of patients are recognized: (1) children, (2) patients who exhibit a slight fever or are not in good condition, (3) patients in good general condition. The smaller initial doses are for patients of the first two groups, the larger for patients in the third group. Other forms of tuberculin are employed, but the types noted above have been given the most extensive trial. Provided reactions are absent or very slight, the injections may be repeated every three or four days. Tuberculin has been given by mouth, but is absorbed irregularly and may produce unexpected reactions. It has also been administered intrafocally in tuberculous pleurisy, tuberculous peritonitis, lupus and tuberculosis of the joints and of the tunica vaginalis. Results have in some instances been encouraging. The local reactions include pain, tenderness and swelling. General reactions are exhibited by rise in temperature, malaise, headache, insomnia, rapid pulse, loss of weight.

Shiga has recently reported upon the use of a "serovaccine." This is designed especially for prophylactic injection in those who by virtue of family relations, constitution or other conditions are predisposed to the disease and for early incipient cases. He claims to have obtained excellent results by weekly vaccination with increasing amounts of the serovaccine followed after fifteen injections by two graded doses of living avirulent tubercle bacilli. The method is prophylactic rather than curative.

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